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Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	Examiner: Charanjit Aulakh
Helmut SCHMIDHAMMER et al.	Group Art: 1625
Serial No.: 10/519,388	
Filed: December 23, 2004	
For: Morphinan Derivatives The Quaternary Ammonium Salts Thereof Substituted In Position 14, Method For Production And Use Thereof	

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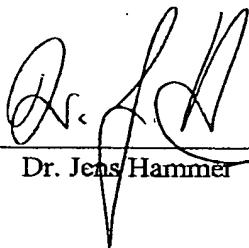### DECLARATION OF DR. JENS HAMMER UNDER 37 C.F.R. §1.132

I, Dr. Jens Hammer, hereby declare as follows:

1.   I am a citizen of Germany, residing at 81667 Muenchen, Woerthstrasse 27, Germany.

2.   I am a European Patent Attorney with the German firm of Grünecker Kinkeldey Stockmair & Schwanhäusser.

3.   I am a native speaker of German, and am fluent, in relevant part, in both written German and English, including scientific German and English.

4.   I have reviewed (i) claim 1 as filed in German in parent application PCT/EP2003/006866 ("German claim 1"; Exhibit 1), and (ii) claim 1 as it appears in the English translation thereof filed with the U.S. Patent and Trademark Office as the subject national stage application ("English claim 1"; Exhibit 2).

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5.   The second and third provisos of German claim 1 recite the phrase "R_4 Wasserstoff (..) ist" which, when translated into English, means "R_4 is hydrogen."

6.   The phrase "R_4 is oxygen", which appears in the second and third provisos of English claim 1, constitutes an erroneous translation of the German phrase " R_4 Wasserstoff (..) ist ".

I further declare that all statements made herein of my own knowledge are true and that all statements are made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____   Dated: _23/2/07_____
    Dr. Jens Hammer

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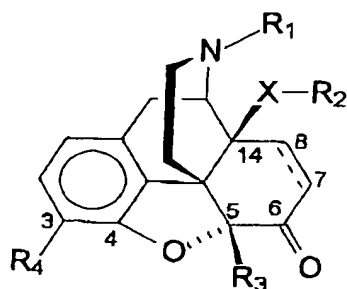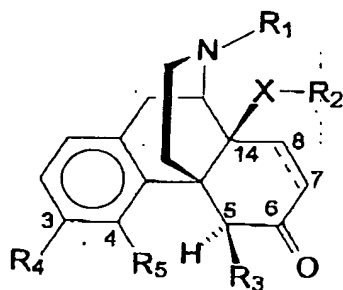Exhibit 1   104

Ansprüche

1.   Verbindungen der Formel (I) oder (Ia),

(I)   (Ia)

in der die Substituenten die folgende Bedeutung haben:

R_1: C_1-C_6-Alkyl; C_2-C_6-Alkenyl; C_2-C_6-Alkinyl; C_3-C_{16}-(cyclische gesättigte Gruppe)alkyl, worin Alkyl C_1-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkenyl, worin Alkenyl C_2-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkinyl, worin Alkinyl C_2-C_6 ist; C_7-C_{16}-Arylalkyl, worin Aryl C_6-C_{10}-Aryl ist und Alkyl C_1-C_6-Alkyl ist; C_8-C_{16}-Arylalkenyl, worin Aryl C_6-C_{10}-Aryl und Alkenyl C_2-C_6-Alkenyl ist; C_8-C_{16}-Arylalkinyl, worin Aryl C_6-C_{10}-Aryl ist und Alkinyl C_2-C_6-Alkinyl;

R_2: vorbehaltlich der folgenden Definition von X, Wasserstoff, C_4-C_6-Alkyl; C_2-C_6-Alkenyl; C_2-C_6-Alkinyl; C_3-C_{16}-(cyclische gesättigte Gruppe)alkyl, worin Alkyl C_1-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkenyl, worin Alkenyl C_2-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkinyl, worin Alkinyl C_2-C_6 ist; C_7-C_{16}-Arylalkyl, worin Aryl C_6-C_{10}-Aryl ist und Alkyl C_1-C_6-Alkyl ist; C_8-C_{16}-Arylalkenyl, worin Aryl C_6-C_{10}-Aryl und Alkenyl C_2-C_6-Alkenyl ist; C_8-C_{16}-Arylalkinyl, worin Aryl C_6-C_{10}-Aryl ist und Alkinyl C_2-C_6-Alkinyl; C_3-C_6-Alkenoyl; C_3-C_6-Alkinoyl; C_9-C_{16}-Arylalkenoyl, worin Aryl C_6-C_{10}-Aryl ist und Alkenoyl C_3-C_6-Alkenoyl ist; C_9-C_{16}-Arylalkinoyl, worin Aryl C_6-C_{10}-Aryl ist und Alkinoyl C_3-C_6-Alkinoyl ist;

R_3: Wasserstoff; C_1-C_6-Alkyl; C_2-C_6-Alkenyl; C_7-C_{16}-Arylalkyl, worin Aryl C_6-C_{10}-Aryl ist und Alkyl C_1-C_6-Alkyl ist; C_8-C_{16}-Arylalkenyl, worin Aryl C_6-C_{10}-Aryl und Alkenyl C_2-C_6-

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Alkenyl ist; Alkoxyalkyl, worin Alkoxy C_1-C_6-Alkoxy und Alkyl C_1-C_6-Alkyl ist; $CO_2(C_1$-C_6-Alkyl); CO_2H; CH_2OH.

R_4: Wasserstoff; Hydroxy; C_1-C_6-Alkyloxy; C_2-C_{10}-Alkyloxyalkoxy, worin Alkyloxy C_1-C_4 ist und Alkoxy C_1-C_6-Alkyloxy ist; C_2-C_6-Alkenyloxy; C_2-C_6-Alkinyloxy; C_3-C_{16}-(cyclische ge-sättigte Gruppe)alkyloxy, worin Alkyl C_1-C_6 ist; C_4-C_{16}-(cyclische gesättigte Grup-pe)alkenyloxy, worin Alkenyl C_2-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkinyloxy, worin Alkinyl C_2-C_6 ist; C_7-C_{16}-Arylalkyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkyl C_1-C_6-Alkyl ist; C_8-C_{16}-Arylalkenyloxy, worin Aryl C_6-C_{10}-Aryl und Alkenyl C_2-C_6-Alkenyl ist; C_8-C_{16}-Arylalkinyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkinyl C_2-C_6-Alkinyl; C_1-C_6-Alkanoyloxy; C_3-C_6-Alkenoyloxy; C_3-C_6-Alkinoyloxy; C_7-C_{16}-Arylalkanoyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkanoyloxy C_2-C_6-Alkanoyloxy ist; C_9-C_{16}-Arylalkenoyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkenoyloxy C_3-C_6-Alkenoyloxy ist; C_9-C_{16}-Arylalkinoyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkinoyloxy C_3-C_6-Alkinoyloxy ist;

R_5: Wasserstoff; Hydroxy; C_1-C_6-Alkyloxy; C_2-C_{10}-Alkyloxyalkoxy, worin Alkyloxy C_1-C_4 ist und Alkoxy C_1-C_6-Alkyloxy ist; C_2-C_6-Alkenyloxy; C_2-C_6-Alkinyloxy; C_3-C_{16}-(cyclische ge-sättigte Gruppe)alkyloxy, worin Alkyl C_1-C_6 ist; C_4-C_{16}-(cyclische gesättigte Grup-pe)alkenyloxy, worin Alkenyl C_2-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkinyloxy, worin Alkinyl C_2-C_6 ist; C_7-C_{16}-Arylalkyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkyl C_1-C_6-Alkyl ist; C_8-C_{16}-Arylalkenyloxy, worin Aryl C_6-C_{10}-Aryl und Alkenyl C_2-C_6-Alkenyl ist; C_8-C_{16}-Arylalkinyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkinyl C_2-C_6-Alkinyl; C_2-C_6-Alkanoyloxy; C_7-C_{16}-Arylalkanoyloxy, worin Aryl C_6-C_{10}-Aryl ist und Alkanoyloxy C_2-C_6-Alkanoyloxy ist;

X ist Sauerstoff, Schwefel oder Methylen;
wobei zwischen den Kohlenstoffatomen der Nummern 7 und 8 eine Einfach- oder eine Doppelbindung vorliegen kann,

wobei Alkyl, Alkenyl und Alkinyl jeweils verzweigt oder unverzweigt sein können, Aryl unsubstituiert oder mono-, di- oder trisubstituiert sein kann, jeweils unabhängig, mit Hy-droxy, Halogen, Nitro, Cyano, Thiocyanato, Trifluormethyl, C_1-C_3-Alkyl, C_1-C_3-Alkoxy, CO_2H, $CONH_2$, $CO_2(C_1$-C_3-Alkyl), $CONH(C_1$-C_3-Alkyl), $CON(C_1$-C_3-Alkyl)$_2$, $CO(C_1$-C_3-Alkyl); amino; $(C_1$-C_3-Monoalkyl)amino, $(C_1$-C_3-Dialkyl)amino, C_5-C_6-Cycloalkylamino; $(C_1$-C_3-Alkanoyl)amido, SH, SO_3H, $SO_3(C_1$-C_3-Alkyl), $SO_2(C_1$-C_3-Alkyl), $SO(C_1$-C_3-Alkyl), C_1-C_3-Alkylthio oder C_1-C_3-Alkanoylthio.

wobei -(cyclische gesättigte Gruppe) entweder bevorzugt C_3-C_{10}-Cycloalkyl ist oder eine heterocyclische Gruppe mit 2 bis 9 Kohlenstoffatomen, enthaltend weiter ein oder mehrere Heteroatome,

mit der Ausnahme von Verbindungen worin R_1 Methyl ist, R_2 C_4-C_6-Alkyl ist, R_3 Wasserstoff oder Methyl ist, R_4 Hydroxy oder Methoxy ist und R_5 Hydroxy, Methoxy oder ein an das Kohlenstoffatom in 5-Stellung gebundenes Sauerstoffatom ist, wenn X Sauerstoff ist;

mit der weiteren Ausnahme von Verbindungen worin R_1 Cyclopropylmethyl und XR_2 Benzyloxy ist, wenn R_4 Wasserstoff oder Benzyloxy ist und R_5 ein an das Kohlenstoffatom in 5-Stellung gebundenes Sauerstoffatom ist;

mit der weiteren Ausnahme von Verbindungen worin R_1 Cyclopropylmethyl und XR_2 Benzyloxy ist, wenn R_4 Wasserstoff, Hydroxy oder Benzyloxy ist und R_5 Hydroxy oder Methoxy ist.

2.   Verbindungen der Formel (IA) oder (IAa),
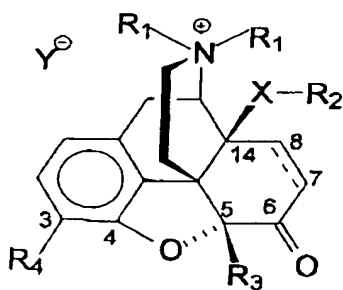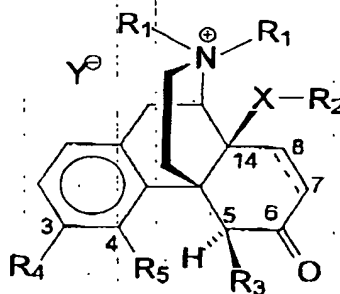
(IA)   (IAa)

worin die Substituenten die folgende Bedeutung haben:

R_1: C_1-C_6-Alkyl; C_2-C_6-Alkenyl; C_2-C_6-Alkinyl; C_3-C_{16}-(cyclische gesättigte Gruppe)alkyl, worin Alkyl C_1-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkenyl, worin Alkenyl C_2-C_6 ist; C_4-C_{16}-(cyclische gesättigte Gruppe)alkinyl, worin Alkinyl C_2-C_6 ist; C_7-C_{16}-Arylalkyl, worin Aryl C_6-C_{10}-Aryl ist und Alkyl C_1-C_6-Alkyl ist; C_8-C_{16}-Arylalkenyl, worin Aryl C_6-C_{10}-Aryl und Alkenyl C_2-C_6-Alkenyl ist; C_8-C_{16}-Arylalkinyl, worin Aryl C_6-C_{10}-Aryl ist und Alkinyl C_2-C_6-Alkinyl;

Claims

1.   Compounds of the formula (I) or (Ia),
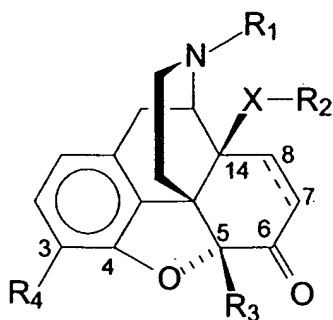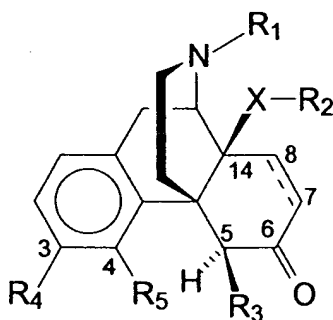
(I)   (Ia)

in which the substituents have the following significance:

R_1: C_1-C_6-alkyl; C_2-C_6-alkenyl; C_2-C_6-alkinyl; C_3-C_{16}-(cyclical saturated group)alkyl, where alkyl is C_1-C_6; C_4-C_{16}-(cyclical saturated group)alkenyl, where alkenyl is C_2-C_6; C_4-C_{16}-(cyclical saturated group)alkinyl, where alkinyl is C_2-C_6; C_7-C_{16}-arylalkyl, where aryl is C_6-C_{10}-aryl and alkyl is C_1-C_6-alkyl; C_8-C_{16}-arylalkenyl, where aryl is C_6-C_{10}-aryl and alkenyl is C_2-C_6-alkenyl; C_8-C_{16}-arylalkinyl, where aryl is C_6-C_{10}-aryl and alkinyl is C_2-C_6-alkinyl;

R_2: subject to the following definition of X, hydrogen, C_4-C_6-alkyl; C_2-C_6-alkenyl; C_2-C_6-alkinyl; C_3-C_{16}-(cyclical saturated group)alkyl, where alkyl is C_1-C_6; C_4-C_{16}-(cyclical saturated group)alkenyl, where alkenyl is C_2-C_6; C_4-C_{16}-(cyclical saturated group)alkinyl, where alkinyl is C_2-C_6; C_7-C_{16}-arylalkyl, where aryl is C_6-C_{10}-aryl and alkyl is C_1-C_6-alkyl; C_8-C_{16}-arylalkenyl, where aryl is C_6-C_{10}-aryl and alkenyl is C_2-C_6-alkenyl; C_8-C_{16}-arylalkinyl, where aryl is C_6-C_{10}-aryl and alkinyl is C_2-C_6-aklinyl; C_3-C_6-alkenoyl; C_3-C_6-alkinoyl; C_9-C_{16}-arylalkenoyl, where aryl is C_6-C_{10}-aryl and alkenoyl is C_3-C_6-alkenoyl; C_9-C_{16}-arylalkinoyl, where aryl is C_6-C_{10}-aryl and alkinoyl is C_3-C_6-alkinoyl;

R_3: hydrogen; C_1-C_6-alkyl; C_2-C_6-alkenyl; C_7-C_{16}-arylalkyl, where aryl is C_6-C_{10}-aryl and alkyl is C_1-C_6-alkyl; C_8-C_{16}-arylalkenyl, where aryl is C_6-C_{10}-aryl and alkenyl is C_2-C_6-alkenyl; alkoxyalkyl, where alkoxy is C_1-C_6-alkoxy and alkyl is C_1-C_6-alkyl; $CO_2(C_1$-C_6-alkyl); CO_2H; CH_2OH.

R_4: hydrogen; hydroxy; C_1-C_6-alkyloxy; C_2-C_{10}-alkyloxyalkoxy, where alkyloxy is C_1-C_4 and alkoxy is C_1-C_6-alkyloxy; C_2-C_6-alkenyloxy; C_2-C_6-alkinyloxy; C_3-C_{16}-(cyclical saturated group)alkyloxy, where alkyl is C_1-C_6; C_4-C_{16}-(cyclical saturated group)alkenyloxy, where alkenyl is C_2-C_6; C_4-C_{16}-(cyclical

saturated group)alkinyloxy where alkinyl is C_2-C_6; C_7-C_{16}-arylalkyloxy, where aryl is C_6-C_{10}-aryl and alkyl is C_1-C_6-alkyl; C_8-C_{16}-arylalkenyloxy, where aryl is C_6-C_{10}-aryl and alkenyl is C_2-C_6-alkenyl; C_8-C_{16}-arylalkinyloxy, where aryl is C_6-C_{10}-aryl and alkinyl is C_2-C_6-alkinyl; C_1-C_6-alkanoyloxy; C_3-C_6-alkenoyloxy; C_3-C_6-alkinoyloxy; C_7-C_{16}-arylalkanoyloxy, where aryl is C_6-C_{10}-aryl and alkanoyloxy is C_2-C_6-alkanoyloxy; C_9-C_{16}-arylalkenoyloxy, where aryl is C_6-C_{10}-aryl and alkenoyloxy is C_3-C_6-alkenoyloxy; C_9-C_{16}-arylalkinoyloxy, where aryl is C_6-C_{10}-aryl and alkinoyloxy is C_3-C_6-alkinoyloxy;

R_5: hydrogen; hydroxy; C_1-C_6-alkyloxy; C_2-C_{10}-alkyloxyalkoxy, where alkyloxy is C_1-C_4 and alkoxy is C_1-C_6-alkyloxy; C_2-C_6-alkenyloxy; C_2-C_6-alkinyloxy; C_3-C_{16}-(cyclical saturated group)alkyloxy, where alkyl is C_1-C_6; C_4-C_{16}-(cyclical saturated group)alkenyloxy, where alkenyl is C_2-C_6; C_4-C_{16}-(cyclical saturated group)alkinyloxy, where alkinyl is C_2-C_6; C_7-C_{16}-arylalkyloxy, where aryl is C_6-C_{10}-aryl and alkyl is C_1-C_6-alkyl; C_8-C_{16}-arylalkenyloxy, where aryl is C_6-C_{10}-aryl and alkenyl is C_2-C_6-alkenyl; C_8-C_{16}-arylalkinyloxy, where aryl is C_6-C_{10}-aryl and alkinyl is C_2-C_6-alkinyl; C_2-C_6-alkanoyloxy; C_7-C_{16}-arylalkanoyloxy, where aryl is C_6-C_{10}-aryl and alkanoyloxy is C_2-C_6-alkanoyloxy;

X is oxygen, sulphur or methylene;
wherein a single or double bond can be present between the carbon atoms of numbers 7 and 8,

wherein alkyl, alkenyl and alkinyl can each be branched or unbranched, aryl can be unsubstituted or mono-, di- or trisubstituted, independently in each case, with hydroxy, halogen, nitro, cyano, thiocyanato, trifluoromethyl, C_1-C_3-alkyl, C_1-C_3-alkoxy, CO_2H, $CONH_2$, $CO_2(C_1$-C_3-alkyl), $CONH(C_1$-C_3-alkyl), $CON(C_1$-C_3-alkyl)$_2$, $CO(C_1$-C_3-alkyl); amino; $(C_1$-C_3-monoalkyl)amino, $(C_1$-C_3-dialkyl)amino; C_5-C_6-cycloalkylamino, $(C_1$-C_3-alkanoyl)amido, SH, SO_3H, $SO_3(C_1$-C_3-alkyl), $SO_2(C_1$-C_3-alkyl), $SO(C_1$-C_3-alkyl), C_1-C_3-alkylthio or C_1-C_3-alkanoylthio,

wherein -(cyclical saturated group) is either preferably C_3-C_{10}-cycloalkyl or a heterocyclical group with 2 to 9 carbon atoms, containing further one or more heteroatoms,

with the exception of compounds where R_1 is methyl, R_2 is C_4-C_6-alkyl, R_3 is hydrogen or methyl, R_4 is hydroxy or methoxy and R_5 is hydroxy, methoxy or an oxygen atom bound to the carbon atom in the 5th position, when X is oxygen;
with the further exception of compounds where R_1 is cyclopropylmethyl and XR_2 is benzyloxy, when R_4 is oxygen or benzyloxy and R_5 is an oxygen atom bound to the carbon atom in the 5th position;
with the further exception of compounds where R_1 is cyclopropylmethyl and XR_2 is benzyloxy, when R_4 is oxygen, hydroxy or benzyloxy and R_5 is hydroxy or methoxy.

Effects of Subcutaneous Methylnaltrexone on Morphine-Induced Peripherally Mediated Side Effects: A Double-Blind Randomized Placebo-Controlled Trial

CHUN-SU YUAN, GANG WEI, JOSEPH F. FOSS, MICHAEL O'CONNOR, THEODORE KARRISON, and JOACHIM OSINSKI

Committee on Clinical Pharmacology (C-S.Y., G.W., J.F.F.), Department of Anesthesia and Critical Care (C-S.Y., G.W., J.F.F., M.O'C., J.O.), and Department of Health Studies (T.K.), University of Chicago, Chicago, Illinois

ABSTRACT

Methylnaltrexone, the first peripheral opioid receptor antagonist, has the potential to prevent or reverse opioid-induced peripherally mediated side effects without affecting analgesia. In previous human trials, we demonstrated that intravenous methylnaltrexone prevented morphine-induced delay in gastrointestinal transit time. We also observed that the compound decreased some of the morphine-induced troublesome subjective effects. However, the effects of subcutaneous methylnaltrexone, a more convenient route of administration, have not been evaluated. In this controlled trial, we evaluated the efficacy of subcutanous methylnaltrexone in antagonizing morphine-induced delay in oral-cecal transit time. In addition, opioid-induced unpleasant subjective effects and pharmacokinetics were studied. We observed that in the first group (n = 6) morphine (0.05 mg/kg intravenously) increased the transit time from a baseline level of 85 ± 20.5 min to 155 ± 27.9 min (mean ± S.D., P < 0.01). After 0.1 mg/kg subcutaneous methylnaltrexone plus morphine, the transit time reduced to 110 ± 41.0 min. In the second group (n = 6), morphine increased the transit time from a baseline level of 98 ± 49.1 min to 140 ± 58.2 min (P < 0.01). After 0.3 mg/kg subcutaneous methylnaltrexone plus morphine, the transit time reduced to 108 ± 59.6 min (P < 0.05 compared with placebo plus morphine). In addition, subcutaneous methylnaltrexone significantly decreased morphine-induced subjective rating changes. Pharmacokinetic data after subcutaneous drug injection were compared to the data obtained from previous intravenous and oral administrations. Our results suggest that subcutaneous methylnaltrexone may have clinical utility in treating opioid-induced constipation and reducing opioid-induced unpleasant subjective symptoms.

Opioid compounds, which are widely administered for a variety of medical indications, are associated with a number of side effects, including constipation and troublesome subjective effects (e.g., dysphoria, dizziness, nausea, and pruritus). Clinically, it would be desirable to reduce opioid-induced peripherally mediated side effects, while maintaining centrally mediated analgesic effect. Selective antagonism of opioid-induced side effects by tertiary compounds such as naloxone or nalmephene have been attempted. Success has been limited by the propensity for these compounds to reverse analgesia or to induce opioid withdrawal (Gowan et al., 1988; Sykes, 1991; Culpepper-Morgan et al., 1992; Cheskin et al., 1995).

N-Methylnaltrexone bromide (or methylnaltrexone) is a

quaternary derivative of the pure opioid antagonist, naltrexone (Brown and Goldberg, 1985). The addition of the methyl group at the amine in its ring forms a compound with greater polarity and lower lipid solubility. Thus, methylnaltrexone does not cross the blood-brain barrier in humans (Russell et al., 1982; Brown and Goldberg, 1985). These properties provide methylnaltrexone with the potential to block undesired side effects of opioid pain medications predominantly mediated by peripherally located receptors (Tavani et al., 1980; Manara et al., 1986), while sparing centrally mediated analgesic effect.

In previous human volunteer trials, we demonstrated that intravenous methylnaltrexone prevented morphine-induced delay in gastrointestinal motility and transit time without affecting analgesia (Yuan et al., 1996). In another preliminary observation, we observed that the compound reduced morphine-induced troublesome subjective effects, such as nausea, skin itch, stimulation, and flushing (Yuan et al., 1998). The present study was designed to evaluate the efficacy of subcutaneously administered methylnaltrexone, a

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ABBREVIATIONS: AUC, area under the curve; T_{max}, time to peak plasma concentration.

more convenient route of administration, on morphine-induced changes in gastrointestinal transit time and subjective effects in healthy volunteers. Pharmacokinetic comparisons were also made after intravenous, oral, and subcutaneous methylnaltrexone.

Materials and Methods

Subjects. With approval from the Institutional Review Board at the University of Chicago, eight males (all Caucasians) and four nonpregnant females (two Caucasians and two African Americans) participated and completed this study. Mean age ± S.D. was 24.8 ± 5.9 (range 19–38) years. All subjects were screened with a medical history, physical examination, 12-lead resting ECG, complete blood count with differential and platelet count, blood chemistries (sodium, potassium, chloride, carbon dioxide, creatinine, blood urea nitrogen, total protein, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, alkaline phosphatase, glucose, calcium, bilirubin, and serum albumin), and urinalysis (specific gravity, pH, protein, blood, glucose, reducing substances, ketones, bilirubin, urobilinogen leukophil esterase, nitrite, and a microscopic examination). Urine toxicology screening for use of other drugs was also performed. Subjects with drug abuse disorders or medical contraindications that would prevent them from participating in the study were excluded.

Protocol. After obtaining written informed consent, subjects were admitted for each experimental day (or session) in the morning to the General Clinical Research Center at the University of Chicago Hospitals after fasting from midnight. There were three sessions, each separated by at least 1 week.

Each session lasted approximately 7 h, and each subject received the following drug combinations: I. placebo plus placebo; II. placebo plus morphine (0.05 mg/kg intravenously); III. subcutaneous methylnaltrexone (0.1 mg/kg in six subjects, and 0.3 mg/kg in another six subjects) plus morphine (0.05 mg/kg intravenously). Drug combination I was always given in session 1 and blinded to the subjects to establish a baseline level and exclude those subjects whose transit time could not be adequately assessed by the lactulose hydrogen breath test (Yuan et al., 1997). Drug combinations II and III were given in sessions 2 and 3 in random order, blinded to both subjects and investigators. Order of assignment was prepared using a table of random numbers and sealed in envelopes. Drug preparation and administration was done by staff members who did not participate in subject observation and data acquisition.

At the onset of each session (baseline or time 0), subjects received a subcutaneous injection of methylnaltrexone or placebo (saline) into the inner thigh with a volume of approximately 0.8 ml. At 15 min, intravenous morphine or placebo (saline) was administered over 1 min. In addition, subjects were instructed to ingest 10 g of lactulose suspended in 100 ml of tap water.

Hydrogen Breath Test. In each session, gastrointestinal transit time was assessed by measuring pulmonary hydrogen (Bond and Levitt, 1975; Basilisco et al., 1985, 1987). This method, which was successfully used in our previous methylnaltrexone studies (Yuan et al., 1996, 1997, 2000), is based on the measurement of hydrogen produced in exhaled air when unabsorbable disaccharide (lactulose) is fermented by colonic bacteria. The time between ingestion of lactulose and the rise of hydrogen in the breath represents the oral-cecal transit time.

Subjects had been asked to eat a meal of boiled rice and meat and water ad libitum and to avoid high fiber cereals and other gasforming foods the evening before each session. End-expiratory breath samples were obtained using 750-ml sample collection bags before lactulose ingestion and every 15 min afterward. Breath samples were measured using a Quintron model 12*i* gas chromatography analyzer (Quintron Instrument Co., Menominee Falls, WI). A standardized gas with a hydrogen concentration of 100 ppm was used to calibrate the instrument before each session. Breath samples were

analyzed within 2 h of collection. Values of hydrogen concentration were expressed in parts per million.

The earliest detectable and sustained rise in pulmonary hydrogen excretion, i.e., a sudden rise to the peak or an increase of at least 2 ppm above the baseline maintained and increased in three consecutive samples, indicated that some of the lactulose had reached the cecum (Bond and Levitt, 1975; Read et al., 1985). Hydrogen breath tests were performed until oral-cecal transit time was determined.

Opioid Subjective Effects. A modified opiate adjective checklist reflecting opioid agonist effects was used (Fraser et al., 1961; Zacny et al., 1994; Yuan et al., 1998). This list consisted of 12 items: "flushing", "stimulated", "numb", "drunken", "difficulty in concentrating", "drowsy (sleepy)", "coasting or spaced out", "turning of stomach", "skin itch", "dry mouth", "dizzy", and "nauseous". Subjects were instructed to rate each of these items on a five-point scale from 0 ("not at all") to 4 ("extremely"). The checklist was completed immediately before the onset of the session (baseline or time 0), 5 min after morphine injection (i.e., time 20 min), and 180 min after morphine injection (i.e., time 195 min). After each test, the ratings for the 12 individual items were summed to give a total subjective symptom score.

Blood and Urine Sampling and Analysis. In each session an intravenous catheter was placed for administration of drugs and blood drawing. Vital signs (heart rate and blood pressure) were monitored before and after drug administrations and when venous blood was collected. Venous blood samples were drawn for plasma drug levels at 0, 2, 5, 10, 15, 20, 30, 45, 60, and 90 min and 2, 3, 4, and 6 h. Urine samples during hours 0 to 3 and 3 to 6 were collected to measure the parent compound.

Measurement of Methylnaltrexone Concentrations. Plasma and urine methylnaltrexone levels were determined by high-performance liquid chromatography technique using a previously reported method (Kim et al., 1989; Yuan et al., 1996). The practical limit of detection for plasma samples was approximately 2 ng/ml.

Drugs. Drugs used were N-methylnaltrexone bromide (Mallinckrodt Chemicals, St. Louis, MO), morphine sulfate (Sanofi Winthrop Pharmaceuticals, New York, NY), and lactulose (Duphalac, Solvay Pharmaceuticals, Marietta, GA).

Statistics. Results of oral-cecal transit time and subjective rating before and after administration of different drug combinations were analyzed using the Wilcoxon signed rank test. In all cases, $P < 0.05$ was considered statistically significant.

Results

One Asian male was excluded from the study after session 1 due to a low hydrogen value with no peak (all < 8 ppm) up to 4.0 h after lactulose administration. Hydrogen production requires a colonic bacterial flora capable of fermenting carbohydrate and yielding hydrogen gas. Previous studies showed that 2 to 27% of individuals, like this subject, had no hydrogen production after lactulose ingestion (Bond and Levitt, 1977; Gilat et al., 1978).

Methylnaltrexone Prevents Morphine-Induced Delay in Oral-Cecal Transit Time. Oral-cecal transit time data are presented in Fig. 1. Transit time increased after morphine administration in all 12 subjects. For the group of six subjects who received 0.1 mg/kg subcutaneous methylnaltrexone (Fig. 1A), intravenous morphine significantly increased the transit time from a baseline level of 85 ± 20.5 min (mean ± S.D.) to 155 ± 27.9 min ($P < 0.01$). After methylnaltrexone plus morphine, the transit time decreased to 110 ± 41.0 min. For the group of six subjects who received 0.3 mg/kg subcutaneous methylnaltrexone (Fig. 1B), intravenous morphine significantly increased the transit time from a baseline level of 98 ± 49.1 min to 140 ± 58.2 min ($P < 0.01$).

Fig. 1. Oral-cecal transit time changes according to drug administration (abscissa) in three different sessions. Morphine indicates 0.05 mg/kg intravenous morphine. A, individual oral-cecal transit time (ordinate) of six healthy volunteers. MNTX 0.1 indicates 0.1 mg/kg subcutaneous methylnaltrexone . B, individual oral-cecal transit time of another six healthy volunteers. MNTX 0.3 indicates 0.3 mg/kg subcutaneous methylnaltrexone . In both parts, heavy lines represent the mean.

After methylnaltrexone plus morphine, the transit time decreased to 108 ± 59.6 min ($P < 0.05$ compared with placebo plus morphine). No laxation response was reported by the subjects after each session.

Figure 2 shows dose-related methylnaltrexone effects from this study and from a previous trial with 0.45 mg/kg intravenous methylnaltrexone (Yuan et al., 1996). Whereas 0.45 mg/kg intravenous methylnaltrexone prevented 97% of morphine-induced increase in oral-cecal transit time, 0.3 and 0.1 mg/kg subcutaneous methylnaltrexone prevented 77% and 64% of morphine-induced increase in the transit time, respectively.

Methylnaltrexone Reduces Morphine-Induced Subjective Effects. Five minutes after morphine administration (i.e., at time 20 min), there were significant increases in subjective ratings (Fig. 3, A and B) (both $P < 0.01$ compared with time 0). Five minutes after 0.1 mg/kg (Fig. 3A) and 0.3 mg/kg (Fig. 3B) subcutaneous methylnaltrexone, morphine-induced subjective ratings were significantly reduced ($P < 0.05$ and $P < 0.01$ compared with placebo plus morphine, respectively).

Pharmacokinetics and Safety. Plasma concentrations after two subcutaneous methylnaltrexone doses are provided in Fig. 4. After the administration of 0.1 and 0.3 mg/kg subcutaneous methylnaltrexone, the unchanged compound detected in urine from 0 to 6 h was 51.8% and 47.3%, respectively. This can be compared to 0.45 to 0.64 mg/kg intrave-

nous methylnaltrexone , in which the amount of unchanged drug excreted during the same period of time was approximately 50% (Yuan et al., 1996; Foss et al., 1997). No adverse effects of clinical importance were observed in this study.

Discussion

Constipation is a very common side effect of advanced cancer patients receiving chronic opioid treatment (Walsh, 1984; Glare and Lickiss, 1992). Tertiary opioid receptor antagonists, such as naloxone, naltrexone, and nalmephene, cross the blood-brain barrier and block both the beneficial pain-relieving effect and the side effects of morphine. Although oral naloxone may reverse opioid-induced constipation, the therapeutic index is very narrow, i.e., reversal of the gut effects with naloxone occurred at doses near the reversal of analgesia (Sykes, 1991). Naloxone may also induce opioid withdrawal symptoms (Gowan et al., 1988; Fifield, 1991; Culpepper-Morgan et al., 1992). As a novel quaternary, peripheral opioid receptor antagonist, methylnaltrexone, even at high doses, has not shown reversal of analgesic effect of morphine in humans (Ferritti et al., 1981; Yuan et al., 1996; Foss et al., 1997). No opioid withdrawal symptoms were observed in our recent study in chronic methadone subjects (Yuan et al., 2000), further indicating that methylnaltrexone does not penetrate into the brain in humans.

In a previous healthy volunteer study, a dose of 0.45 mg/kg
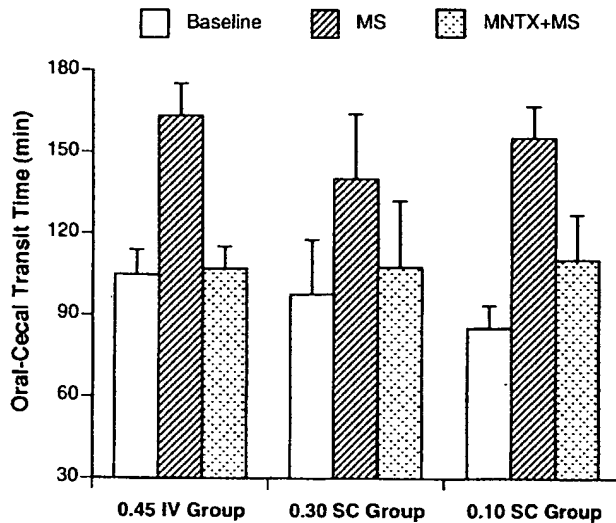
Fig. 2. Dose-related effects of subcutaneous methylnaltrexone compared with 0.45 mg/kg intravenous methylnaltrexone from a previous study in 12 normal subjects or 0.45 IV group (Yuan et al., 1996). 0.30 SC Group, 0.3 mg/kg subcutaneous methylnaltrexone in six subjects. 0.10 SC Group, 0.1 mg/kg subcutaneous methylnaltrexone in another six subjects. 0.45 IV group, 0.30 SC group, and 0.10 SC group prevented 97%, 76%, and 64% of morphine-induced increase in oral-cecal transit time, respectively.

intravenous methylnaltrexone was able to completely reverse morphine-induced changes in gut transit time (Yuan et al., 1996). In this study, a lower subcutaneous methylnaltrexone dose (0.1 mg/kg) was also chosen, because we planned to test this compound in chronic opioid users who were very sensitive to methylnaltrexone compared with normal opioid-naive subjects (Yuan et al., 1999, 2000). Approximately 18% of the dose of intravenous methylnaltrexone (0.08 mg/kg) was needed to reverse chronic opioid-induced constipation compared with a dose of 0.45 mg/kg used in normal subjects (Yuan et al., 1996, 2000). In this study, we observed that 0.3 mg/kg subcutaneous methylnaltrexone significantly prevented morphine-induced delay of oral-cecal transit time in subjects who received a single acute dose of morphine. Among six subjects in the 0.1 mg/kg group, there was a

nonresponder (Fig. 1A). Thus, statistical significance was not achieved due to high variability and small sample size. Since chronic opioid users are very sensitive to opioid antagonist (Yuan et al., 2000), it seems that subcutaneous methylnaltrexone at a dose of 0.1 mg/kg or less will reverse chronic opioid-induced constipation significantly.

In addition to opioid-induced gut side effects, opioid-induced unpleasant feelings, such as dizziness, headache, nausea, dry mouth, warmth, tingling, and itchiness, have long been recognized (Lasagna et al., 1955). In humans, the effects of drugs on subjective responses could be expressed in quantitative terms (Smith and Beecher, 1959, 1962; Fraser et al., 1961; Zacny et al., 1994). In this study, using the modified adjective checklist, which is sensitive to the subjective and somatic effects of μ-class opioid agonists (Preston et al., 1989), we observed that subcutaneous methylnaltrexone significantly reduced the overall subjective effect rating.

Data from previous animal studies showed that heroin-induced "rush" sensation was involved in the opioid receptors located within the central nervous system (Koob et al., 1984) and aversive conditioning effects of morphine were primarily mediated through peripheral opioid receptors (Bechara et al., 1987). Human volunteer study data demonstrated that intravenous methylnaltrexone, a peripheral opioid receptor antagonist, did not reverse morphine-induced centrally mediated analgesic effects (Yuan et al., 1996). While some items in the checklist (e.g., "coasting or spaced out") are believed to occur due to opioid effects on the central nervous system, observations from our previous volunteer studies suggest that a peripheral opioid antagonist reduced some of these subjective effects (Yuan et al., 1998). Several items in the checklist used in this study (e.g., "nauseous", "flushing", "skin itch") may not be centrally mediated symptoms (Levy et al., 1989; Foss et al., 1993; Reisine and Pasternak, 1996). Because of relatively low statistical power (six subjects per group), we summed the 12 individual items in the checklist to obtain a total subjective symptom score, rather than analyzing each individual item.

"Drowsy (sleepy)" perhaps is another centrally mediated opioid effect, and only a slight increase in this rating after morphine was noted in most subjects. However, two subjects
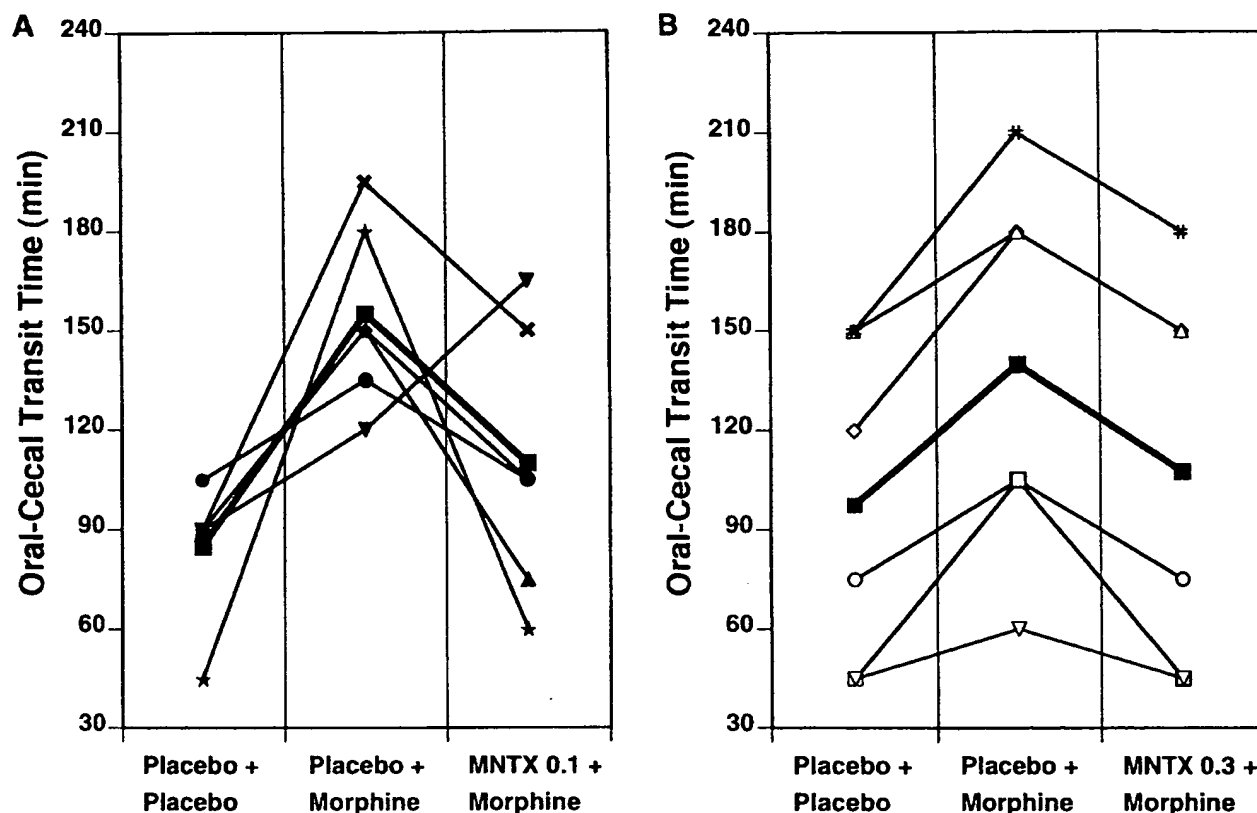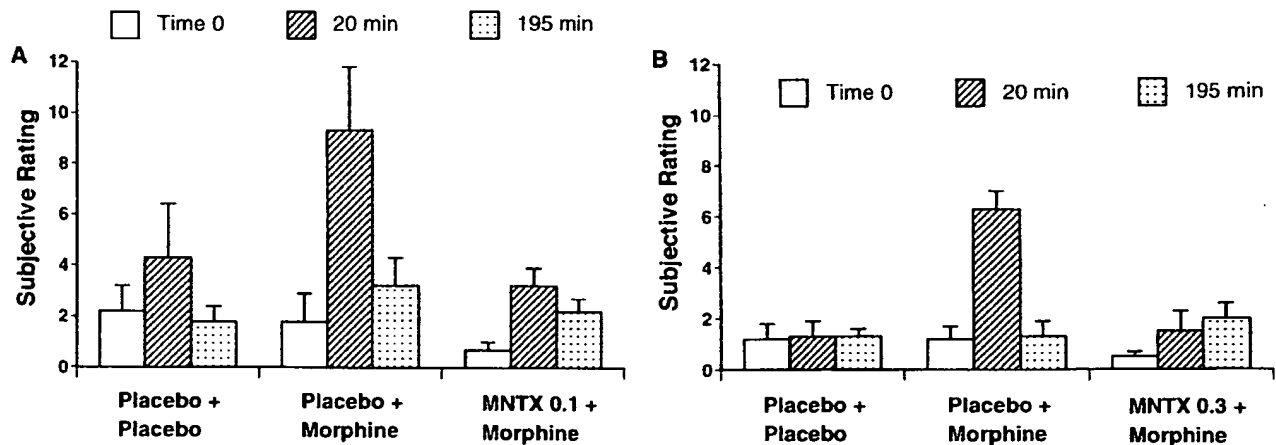

Fig. 3. Subjective ratings according to drug administration (abscissa) in three different sessions. Morphine indicates 0.05 mg/kg intravenous morphine. A, ratings of six subjects in 0.1 mg/kg subcutaneous methylnaltrexone group. B, ratings of another six subjects in 0.3 mg/kg subcutaneous methylnaltrexone group. In each session, the ratings were obtained at baseline or immediately before the onset of the session (Time 0), 5 min after 0.05 mg/kg intravenous morphine injection (Time 20 min), and 180 min after morphine administration (Time 195 min).
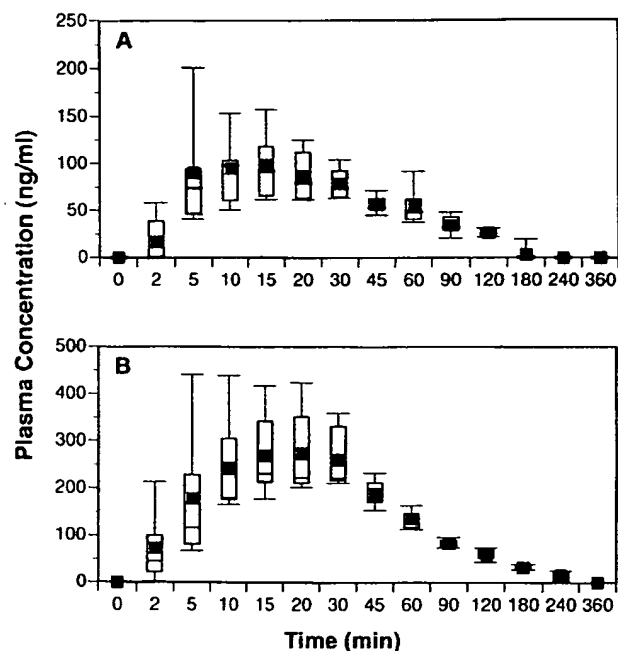
Fig. 4. Box plot of plasma methylnaltrexone concentrations in healthy volunteers after subcutaneous drug injection. A, after 0.1 mg/kg subcutaneous methylnaltrexone (n = 6). B, after 0.3 mg/kg subcutaneous methylnaltrexone (n = 6). Black squares represent the mean values, short horizontal lines represent median values, vertical lines represent 90 and 10 percentile values, respectively, and open boxes represent 75 and 25 percentile values, respectively. Note the same time scale but different concentration scales in parts A and B.

in the 0.1 mg/kg methylnaltrexone group were very sleepy before the onset of two sessions (placebo plus placebo session and placebo plus morphine session), and they marked very high "drowsy (sleepy)" ratings. Since there were only six subjects per group, their selection made the average of these two sessions in Fig. 3A much higher with long error bars. In future experiments, it would be desirable to evaluate the subjective effects in a higher number of subjects, and each item in the checklist can be analyzed separately. The site of action (peripheral versus central) of some subjective symptoms has not been determined yet. Methylnaltrexone can be used as a "probe" to differentiate putatively peripherally mediated or centrally mediated subjective symptoms.

Clinically, subjective effects caused by morphine can cause an unpleasant, troublesome experience. Opioid medications, often given to patients during and after surgical procedures,

may possibly delay postoperative recovery because of these subjective effects. Our data suggest that some or many of these effects may be separable from centrally mediated opioid-induced analgesia. As a peripheral opioid receptor antagonist, methylnaltrexone may facilitate faster recovery, as it may decrease unpleasant effects but still allow faster mobilization while preserving analgesia. It appears that methylnaltrexone may have a potential therapeutic value in decreasing some uncomfortable subjective effects due to opioid medication.

In this study, we also compared pharmacokinetic data for subcutaneous methylnatrexone to those parameters from our past drug trials using different routes of administration. Table 1 presents the pharmacokinetic parameters for subcutaneous methylnatrexone obtained from this study and compares these data to previous results from intravenous methylnaltrexone (Yuan et al., 1996, 2000) and oral methylnaltrexone (Yuan et al., 1997). Peak free plasma concentration is significantly lower after subcutaneous injection compared with intravenous administration. Whereas T_{max} can be reached instantaneously after intravenous dosing, T_{max} is reached at approximately 16 to 20 min after subcutaneous administrations. T_{max} is significantly faster after subcutaneous injection compared with oral medication. The dose difference between oral and subcutaneous routes was approximately 100 times, but the AUC values were not remarkably different. For eight chronic methadone subjects after approximately 0.08 ± 0.04 mg/kg slow intravenous infusion of methylnaltrexone (Yuan et al., 2000), the AUC value was similar compared with those normal subjects who received 0.1 mg/kg subcutaneous drug in this study.

Bioavailability of oral drugs is often erratic and incomplete (Rowland and Tozer, 1995). Since methylnaltrexone is a charged compound (Brown and Goldberg, 1985), gut absorption is particularly limited (Yuan et al., 1997). Values of AUC after oral dose also showed a greater variability among individual subjects probably due to high impedance of absorption in the gut.

Subcutaneous administration provided rapid onset with a total drug effect comparable with intravenous or oral administration. We observed efficacy of subcutaneous methylnaltrexone in this study in preventing morphine-induced delay in oral-cecal transit time. Our data suggest that methylnaltrexone by subcutaneous route brings on the effect more rapidly and reliably than the oral route, while avoiding the maintenance of an intravenous site.

Compared with intravenous medication, subcutaneous ad-

TABLE 1

Pharmacokinetic parameters (mean ± S.D.) for subcutaneous (SC), intravenous (IV), and oral methylnaltrexone

| Drug Route	No. of Subjects	Dose	C_{max}	T_{max}	AUC	$t_{1/2}$	CL	F_u
		mg/kg	ng/ml	min	ng/ml · h	min	l/h	%
SC	6	0.1	110 ± 55	16.7 ± 10.8	132 ± 19	105 ± 27	53.4 ± 13.3	51.7 ± 8.8
SC	6	0.3	287 ± 101	20.0 ± 9.5	367 ± 79	132 ± 19	55.8 ± 8.5	47.3 ± 6.6
IV[a]	12	0.45	3299 ± 103		677 ± 23	131 ± 49	52.5 ± 2.8	47.8 ± 11.5
IV[b]	8	0.08	124 ± 82	26.7 ± 9.4	136 ± 89	129 ± 48	52.2 ± 30	29.1 ± 11.0*
Oral[c]	14	19.2	166 ± 251	116 ± 102	419 ± 388	204 ± 82		0.64 ± 0.4

C_{max}, peak free plasma concentration; AUC, area under the plasma concentration-time curve from 0 to 6 h; $t_{1/2}$, β half-life; CL, total body clearance; F_u, percentage of dose excreted unchanged in urine from 0 to 6 h.

* From 0 to 4 h.
[a] Yuan et al. (1996).
[b] Yuan et al. (2000), using slow intravenous infusion; pharmacokinetic data have not been reported before.
[c] Yuan et al. (1997).

ministration is a more convenient and safer method to deliver drugs (Nucci and Cobelli, 2000; Lepore et al., 2000). In addition, patients with chronic opioid-induced constipation would be able to self-medicate at home, like diabetic patients being able to self-inject insulin subcutaneously. Data from this study showed that subcutaneous methylnaltrexone effectively prevented a single acute dose of morphine-induced gut motility change. In future studies, the dose relationship of agonist to antagonist will be evaluated in opioid-tolerant individuals, such as advanced cancer patients receiving chronic opioid pain medications.

Acknowledgments

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Address correspondence to: Dr. Chun-Su Yuan, Department of Anesthesia and Critical Care, University of Chicago, 5841 S. Maryland Ave., MC 4028, Chicago, IL 60637. E-mail: cyuan@midway.uchicago.edu

Anti-inflammatory properties of the μ opioid receptor support its use in the treatment of colon inflammation

David Philippe,[1] Laurent Dubuquoy,[1] Hervé Groux,[2] Valérie Brun,[2]
Myriam Tran Van Chuoï-Mariot,[3] Claire Gaveriaux-Ruff,[4] Jean-Frédéric Colombel,[1]
Brigitte L. Kieffer,[4] and Pierre Desreumaux[1]

[1]Equipe Mixte INSERM 0114 sur la Physiopathologie des Maladies Inflammatoires Intestinales,
 Centre Hospitalier Universitaire, Lille, France
[2]Unité INSERM U343, and TxCell, Hôpital de l'Archet 1, Nice, France
[3]Unité INSERM U422, Centre Hospitalier Universitaire, Lille, France
[4]Institut de Génétique et de Biologie Moléculaire et Cellulaire, UMR7104, Strasbourg, France

The physiologic role of the μ opioid receptor (MOR) in gut nociception, motility, and secretion is well established. To evaluate whether MOR may also be involved in controlling gut inflammation, we first showed that subcutaneous administration of selective peripheral MOR agonists, named DALDA and DAMGO, significantly reduces inflammation in two experimental models of colitis induced by administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) or peripheral expansion of CD4+ T cells in mice. This therapeutic effect was almost completely abolished by concomitant administration of the opioid antagonist naloxone. Evidence of a genetic role for MOR in the control of gut inflammation was provided by showing that MOR-deficient mice were highly susceptible to colon inflammation, with a 50% mortality rate occurring 3 days after TNBS administration. The mechanistic basis of these observations suggests that the anti-inflammatory effects of MOR in the colon are mediated through the regulation of cytokine production and T cell proliferation, two important immunologic events required for the development of colon inflammation in mice and patients with inflammatory bowel disease (IBD). These data provide evidence that MOR plays a role in the control of gut inflammation and suggest that MOR agonists might be new therapeutic molecules in IBD.

Introduction

The endogenous opioid peptide β-endorphin and opiate compounds such as morphine have well-known central and peripheral analgesic effects through activation of the μ opioid receptor (MOR) (1, 2). Since MOR agonists exert inhibitory effects on intestinal motility and secretion (3-5), opioids are also widely used in the symptomatic treatment of diarrhea (6, 7). Besides these classical therapeutic properties of opioid drugs, the demonstration of opioid peptide and MOR expression by cells involved in the inflammatory response (8-11) has led to new investigations showing the roles of MOR modulators in the regulation of the immune system and inflammatory reactions (12-17).

MOR, a member of the G protein–linked receptor superfamily (18), is found in the central (19, 20) and peripheral nervous system (21, 22). This receptor is expressed in various tissues including the gut, particularly on lymphocytes (23) and myenteric and submucosal plexi (24). In vitro studies have shown that both opioids and naloxone, an opioid receptor antagonist, may modulate mitogen-induced PBMCs (25), splenocyte proliferation (26, 27), NK cell activity (28, 29), and production of inflammatory (30-32) and immunoregulatory cytokines (17, 33–35). In vivo evidence of the regulatory immune functions of MOR activators has also been reported in several animal models of autoimmune (36, 37) and inflammatory diseases (38-40).

While there is clear evidence for potential therapeutic roles of MOR ligands in the treatment of inflammatory bowel disease (IBD), the anti-inflammatory effects of selective MOR activators during intestinal inflammation remain unexplored. In the present study, we first investigated the potential effects of selective MOR agonists and one antagonist in the experimental animal model of colitis induced by intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) (41) and by peripheral expansion of CD45RB^hi T cells transferred into immunodeficient SCID mice (42). These two different models are well described and share many macroscopic and histologic similarities with IBD, including ulcerations, granulomas, transmural inflammation with neutrophil infiltrates, and upregulation of the TNF-α signaling pathway (43, 44). We also examined the genetic involvement of MOR in colon inflam-

mation by studying the consequences of TNBS administration in MOR knockout ($MOR^{-/-}$) mice. Next, to further define the mechanistic basis of our observations, we evaluated the influence of MOR activation on the production of cytokines and expansion of T cells, two essential immunologic events required for the development and progression of colitis (45–48).

We describe an intestinal anti-inflammatory activity of MOR agonists and a dramatic increase of inflammation and mortality in $MOR^{-/-}$ mice. Experiments addressing the mechanisms for the abrogation of colitis indicate the regulatory roles of MOR on cytokine production and T cell proliferation. In addition to their analgesic and antidiarrheic effects, MOR agonists therefore may be an alternative to the traditional therapeutic approaches to IBD, chronic intestinal disorders characterized by inflammation, pain, and diarrhea.

Methods

The MOR agonists [D-Arg^2,Lys^4]dermorphin-(1,4)-amide (DALDA) (49) and [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) (50, 51), and the general opioid antagonist naloxone methiodide (NM) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). TNBS was purchased from Sigma-Aldrich.

Induction of TNBS colitis and study design. Animal experiments were performed in accredited establishments at the Institut de Génétique et de Biologie Moléculaire et Cellulaire from Strasbourg and at the Institut Pasteur from Lille according to governmental guidelines. Animals were housed five per cage and had free access to standard mouse chow and tap water. For colitis induction, mice were anesthetized for 90–120 minutes and received an intrarectal administration of TNBS (40 μl, 150 mg/kg) dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol (41). Control mice received a 1:1 mixture of 0.9% NaCl with 100% ethanol or a saline solution using the same technique. Animals were sacrificed 2 days or 4 days after TNBS administration (41). The anti-inflammatory effects of MOR agonists in Balb/c mice were evaluated by administration of different dosages of DALDA (10^{-3} to 1 mg/kg/d) and DAMGO (10^{-3} to 0.5 mg/kg/d), which selectively activate peripheral MOR and lack the ability to cross the blood-brain barrier (Figure 1) (52, 53). These compounds were administered once daily by subcutaneous injection, starting either 4 days before (preventive mode) or 30 minutes after (treatment mode) colitis induction. To determine whether the beneficial effects of DALDA and DAMGO were due to selective activation of peripheral MOR, the MOR antagonist NM, which does not cross the blood-brain barrier (54), was given in some mice preventively and concomitantly 6 days before colitis induction at the dose of 2.5 mg/kg/d as described previously (55). Macroscopic, histological, and biological assessments of colitis were performed blindly by two investigators. In a second set of experiments, genetic evidence for the involvement of MOR in colon inflammation was evaluated by the induction of colitis in $MOR^{-/-}$ mice (56) that were backcrossed for ten

generations onto C57BL/6 mice and their wild-type littermates (9.9 ± 0.88 weeks of age). Due to higher susceptibility of these knockout mice to inflammation, animals were sacrificed 3 days after colitis induction.

Macroscopic and histologic assessment of TNBS-induced colitis. The colon of each mouse was examined under a dissecting microscope (magnification, ×5) to evaluate the macroscopic lesions according to the Wallace criteria. The Wallace score rates macroscopic lesions on a scale from 0 to 10 based on features reflecting inflammation, such as hyperemia, thickening of the bowel, and extent of ulceration (57). A colon specimen located precisely 2 cm above the anal canal was cut into three parts. One part was fixed in 4% paraformaldehyde and embedded in paraffin. Sections stained with May-Grunwald-Giemsa were examined blindly by two investigators and scored according to the Ameho criteria (58). This grading on a scale from 0 to 6 takes into account the degree of inflammation infiltrate, the presence of erosion, ulceration, or necrosis, and the depth and surface extension of lesions (58). The other two parts of the colon were frozen and used to quantify myeloperoxidase (MPO) and mRNA of inflammatory cytokines.

Quantification of MPO. Protein preparation and immunoblotting were performed as described (59). Total protein extracts were obtained by homogenization of colon samples in a PBS lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (59). Total proteins (50 μg) were separated by PAGE and electroblotted (59). Immunodetection of MPO was performed after an overnight incubation of the immunoblotted membrane with a rabbit polyclonal primary antibody (dilution 1:500; DAKO Corp., Trappes, France) at 4°C. Then a swine secondary peroxidase-conjugated antibody (dilution 1:1,000, DAKO Corp.) was added for 1 hour at room temperature. The complex was detected by chemiluminescence according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech, Orsay, France). Results were expressed as units of OD per 50 ng of total protein.

Reconstitution of SCID mice with T cell subpopulations and treatment with MOR agonists. Intestinal inflammation was induced in 6-week-old CB-17 SCID mice by intraperitoneal injection of 100 μl of PBS containing 2.5×10^5 CD4$^+$CD45RB^{hi} T cells (44). Control of this experiment was provided by coinjection into the same mice of 1.25×10^5 CD4$^+$CD45RB^{lo} T cells and 2.5×10^5 CD4$^+$CD45RB^{hi} T cells (44). These two CD4$^+$ T cell subsets were purified from the spleens of immunocompetent mice as previously described with the following modifications (44, 60, 61). Briefly, cells were depleted of B220$^+$, Mac-1$^+$, I-Ad$^+$, and CD8$^+$ cells by negative selection using sheep anti-rat–coated antibody Dynabeads (Dynal Biotech, Oslo, Norway). The remaining cells were labeled with FITC-conjugated anti-CD45RB (25 μg/ml) and phycoerythrin-conjugated (PE-conjugated) anti-CD4 (10 μg/ml) and separated into CD4$^+$CD45RB^{hi} and CD4$^+$CD45RB^{lo} fractions by two-color sorting on a FACStar plus cytometer/sorter (Becton, Dickinson and

Co., Le Pont de Claix, France). All populations were more than 98% pure on repeat analysis.

The anti-inflammatory effects of MOR agonists in CB-17 SCID mice were evaluated by subcutaneous administration of the optimal dosage of DALDA (10^{-2} mg/kg/d) or DAMGO (10^{-3} mg/kg/d). These compounds were administered the day after reconstitution and continued once daily for the 4-week duration of the experiment, before mice were killed.

Clinical and microscopic examination of SCID mice. T cell-restored SCID mice were weighed weekly and sacrificed after 4 weeks. Disease development in CD45RB^{hi} T cell–reconstituted SCID mice was evaluated by analysis of weight loss and mortality rates, measurements of splenomegaly and colon thickness, and histologic assessment of the colon. For the microscopic examination, a 1-cm piece of the distal colon was removed and embedded in paraffin. Sections were stained with May-Grunwald-Giemsa stain and graded semiquantitatively from 0 to 3 in a blinded fashion (62, 63). A grade of 0 was given when there were no changes observed. Changes typically associated with other grades were as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and loss of goblet cells; grade 3, massive and extensive leukocytic infiltrations that were sometimes transmural, often associated with ulceration, epithelial hyperplasia, and mucin depletion. The other parts of the distal colon were used to purify lamina propria lymphocytes and to quantify mRNA of inflammatory cytokines.

Isolation of CD4⁺ T cells from spleen and colon of T cell–restored SCID mice. Four weeks after T cell transfer, CD4⁺ lymphocytes were isolated from the spleen and colon as described previously, with some modification (44). Splenocytes were counted and labeled with FITC-conjugated anti-CD4 and PE-conjugated anti-CD3 (10 μg/ml; Pharmingen, Le Pont de Claix France) and the number of CD4⁺ T cells was determined by FACS analysis (64). For the colon, samples were washed in a solution of RPMI 1640 containing antibiotics and cut into 0.5-cm pieces. Mucosal layers were dissected away from the muscular and serosal layers and incubated in RPMI containing 1 mM DTT. Mucosal pieces were minced and stirred in calcium- and magnesium-free HBSS supplemented with heat-inactivated FCS (2%) and 1 mM EDTA. Lamina propria lymphocytes were isolated after incubating mucosal pieces with 1 mg/ml of collagenase-dispase (Sigma-Aldrich) for 90–180 minutes at 37°C. Lymphocytes were recuperated by filtration of the supernatant and CD4⁺ T cell number was determined by FACS analysis.

Quantification of cytokines and MOR mRNA in the colon. RNA was isolated from colon samples with TRIzol reagent as described (65). After treatment at 37°C for 30 minutes with 20–50 units of DNase I RNase-free (Roche Molecular Biochemicals, Mannheim, Germany), total RNA (5–10 μg) was reverse-transcribed into cDNA. The reverse transcription reaction mixture was amplified by quantitative PCR using primers and competitors specific for β-actin, TNF-α, IL-1β, IL-4, and IFN-γ (41, 66). Samples and competitors were subjected to 40 PCR cycles (PE Corp., Foster City, California, USA). Quantification of cDNA was performed by electrophoresis in 3% agarose gel using an image analyzer (Gel Analyst; Clara Vision, Paris, France) (67). The results were expressed as number of mRNA molecules per 10^4 mRNA molecules of an internal control, β-actin, in the same sample. MOR mRNA expression was also evaluated by RT-PCR using selected primers in proportion to a known number of β-actin mRNA molecules in the same sample (68).

Statistics. Data were expressed as mean ± SEM. All comparisons were analyzed by the nonparametric Kruskal-Wallis one-way ANOVA test or by the Mann-Whitney U test. Statistical analyses were performed using the StatView 4.5 statistical program (SAS Institute Inc., Meylan, France). Differences were considered significant when the P value was below 0.05.

Results

Attenuation of TNBS-induced colitis by administration of MOR agonists. First we characterized the development of colitis in animals subjected to TNBS injection. Whereas control mice sacrificed 2 days or 4 days after administration of 50% ethanol or a saline solution showed no macroscopic or histologic lesions in the colon, an acute colitis was induced as early as 2 days after TNBS administration, resulting in death in 48% of the Balb/c mice. Four days after colitis induction, the lesions were more severe, showing necrosis of the colon and leading to
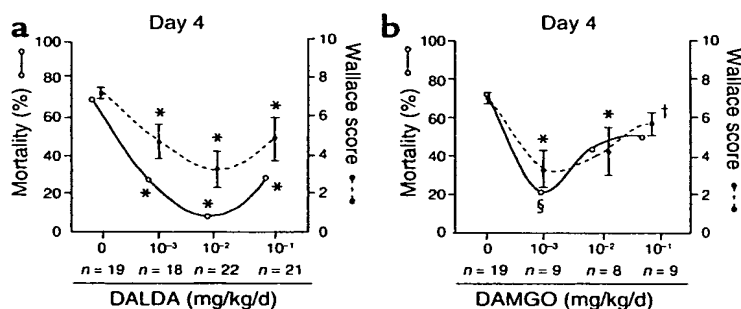
Figure 1
Dose-response study of the effects of DALDA and DAMGO on TNBS-induced colitis. The anti-inflammatory effects of different doses of DALDA (**a**) and DAMGO (**b**) given in preventive mode, once daily by subcutaneous administration, were assessed in mice sacrificed 4 days after TNBS administration. Colitis severity was evaluated by mortality rates, expressed as percentages (open circles), and by the intensity of macroscopic lesions (filled circles, mean ± SEM), assessed using the Wallace score. The different doses of agonists and the number of mice receiving each dosage are indicated. *$P < 0.001$, §$P < 0.01$, and †$P < 0.05$ in treated mice versus untreated mice with colitis.

a Morphologic examination

Control   TNBS   DALDA + TNBS

b Macroscopic scores
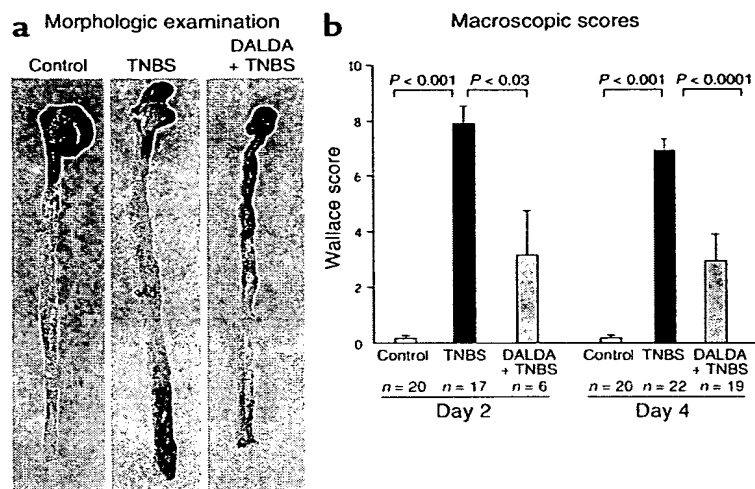
Figure 2
DALDA improves the macroscopic lesions induced by TNBS. (a) Morphologic examination of mice receiving vehicle only (control), TNBS, or DALDA (10^{-2} mg/kg/d) 4 days prior to the administration of TNBS (DALDA + TNBS). (b) The anti-inflammatory effects of DALDA (10^{-2} mg/kg/d) were assessed preventively 2 days (day 2) and 4 days (day 4) after TNBS administration. Severity of lesions was evaluated using the macroscopic Wallace score. The number of mice and statistical significance are indicated and results are expressed as mean ± SEM.

mortality in 73% of the Balb/c mice (Figure 1 and Figure 2). TNBS-induced colitis was characterized by hyperemia of the mucosa with large areas of ulceration and thickening of the bowel wall (Figure 2). These lesions were associated with neutrophilic infiltration extending from the mucosa deep into the muscular layer and with an enhanced concentration of colon MPO, a marker of neutrophil content (69) (Figure 3).

Since DALDA and DAMGO exert their biological actions via MOR, we first verified that MOR mRNA was expressed in the healthy (MOR mRNA OD, 2.5 ± 0.5 measured in 10^4 β-actin molecules) colon of mice, with increased levels during inflammation (MOR mRNA OD, 5.2 ± 1.1 measured in 10^4 β-actin molecules, $P < 0.02$). We then evaluated the effects of MOR activation on mortality rates and macroscopic inflammatory scores by performing a detailed dose-response study using the synthetic MOR agonists DALDA (from 10^{-3} to 1 mg/kg/d) and DAMGO (from 10^{-3} to 0.5 mg/kg/d), administered preventively 4 days before colitis induc-

tion, once daily until sacrifice (Figure 1). Two days and 4 days after TNBS administration, DALDA- and DAMGO-treated mice showed significantly reduced mortality compared with untreated mice with colitis, and had improved macroscopic lesions (Figure 1 and Figure 2). Similar beneficial effects were seen at 2 days and 4 days after TNBS administration with the doses of 10^{-2} mg/kg/d DALDA and 10^{-3} mg/kg/d DAMGO (Figure 1). Parallel to the gross macroscopic inflammation, histologic analysis also revealed major improvement of colitis in animals treated with DALDA compared with untreated mice (Figure 3). This was reflected by a reduction of the neutrophil infiltrate, which was limited to the mucosa, and was associated with a significant decrease of MPO levels, an absence of ulceration, and a normalization of colon wall thickness, leading to a significant decrease of the Ameho inflammatory score evaluated both at 2 days and at 4 days after TNBS administration (Figure 3).

In all the experiments described above, MOR agonists were given in preventive mode before colitis induction. We next analyzed whether the administration of DALDA after the induction of colitis was also effective in reducing the intensity of inflammation. DALDA administered after colitis induction significantly

Figure 3
DALDA improves the histologic lesions and decreases the MPO level induced by TNBS. (a–c) Histologic examination of colon sections of mice receiving vehicle only, TNBS, or 10^{-2} mg/kg/d DALDA followed 4 days later by TNBS (DALDA + TNBS) (magnification, ×250). TNBS induced severe lesions characterized by an intense inflammatory infiltrate extending deep into the colon wall with necrosis (b). Treatment with DALDA reduced the intensity of histologic inflammation, which was characterized only by a moderate inflammatory infiltrate located in the mucosa (c). (d) The histologic anti-inflammatory effects of DALDA (10^{-2} mg/kg/d) were assessed preventively 2 days and 4 days after TNBS administration. Colon MPO levels were evaluated 4 days after colitis induction. Severity of lesions was evaluated using the histologic scoring method of Ameho. The number of mice and statistical significance are indicated and results are expressed as mean ± SEM.
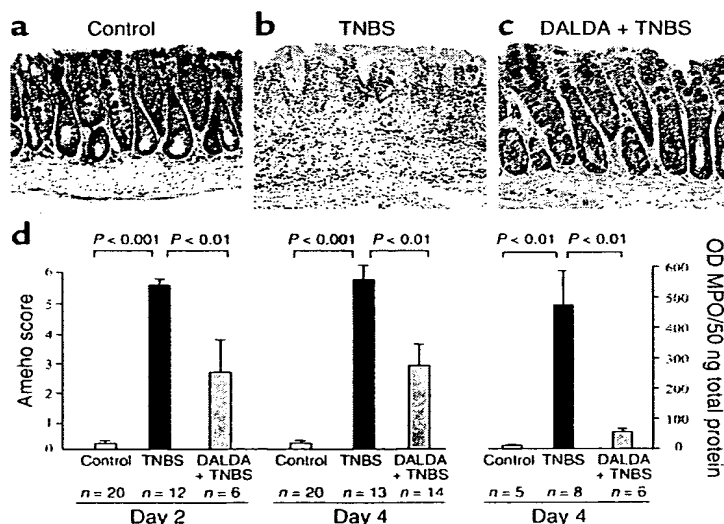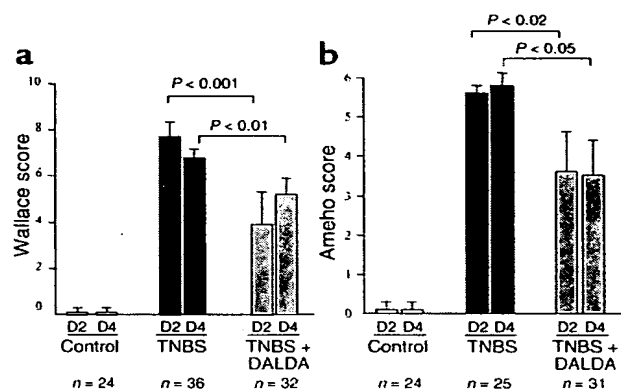
Figure 4
DALDA administered in treatment mode improves the macroscopic and histologic lesions. (a and b) The anti-inflammatory effects of DALDA (10^{-2} mg/kg/d) administered in treatment mode (after colitis induction) were assessed 2 days (D2) and 4 days (D4) after TNBS administration. The severity of the lesions was evaluated using the macroscopic and histologic scores of Wallace (a) and Ameho (b), respectively. The number of mice and statistical significance are indicated and results are expressed as the mean ± SEM.

reduced mortality (19% vs. 45% at day 2 and 40% vs. 73% at day 4) and macroscopic and histologic scores 2 days and 4 days after TNBS administration (Figure 4). These data demonstrate that MOR agonists can not only prevent lesion development but are also effective in reducing established inflammatory lesions in the colon.

The opioid antagonist NM abolishes the anti-inflammatory effects of DALDA. As expected, administration of NM blocked the protective effects of DALDA as shown by the similar macroscopic and histologic lesions and MPO levels found in untreated mice with colitis and mice with colitis simultaneously receiving NM and DALDA (Figure 5). Moreover, compared with untreated animals with colitis, animals administered NM alone showed increased inflammation 4 days after TNBS administration, with more numerous and deeper colon ulcerations and an enhancement of MPO levels (data not shown). Taken together, these data further confirm that the anti-inflammatory effect of DALDA is mediated by peripheral MOR activation.

Increased susceptibility of MOR$^{-/-}$ mice to TNBS-induced colitis. As the data from DALDA and DAMGO treatment indicate that an interaction between agonists and MOR regulates colon inflammation, we next tested whether mice homozygous for a deficiency of MOR were more susceptible to TNBS-induced colitis. MOR$^{-/-}$ mice did not differ from their littermates in general health (56) (Figure 6, a–e) and had normal stools. No morphologic or histologic intestinal abnormalities were detected in MOR$^{-/-}$ mice compared with controls (Figure 6). Relative to Balb/c mice, TNBS induced less severe colitis in wild-type C57BL/6 mice, as shown by the comparison of the mortality rate and macroscopic and histologic scores (Figure 1, Figure 2, Figure 3, and Figure 6). Although no spontaneous macroscopic or histologic inflammation

was found in the colon of MOR$^{-/-}$ mice, these knockout animals developed rapidly lethal colitis, reaching a mortality rate of 50% 3 days after TNBS administration, while wild-type mice displayed 17% mortality (Figure 6a). The lesions were severe in MOR$^{-/-}$ mice compared with those in control mice, involved the whole length of the colon, and were characterized by mucosal necrosis and perforation (Figure 6). In combination with the data indicating that MOR agonists significantly reduce colon inflammation, this study in MOR$^{-/-}$ mice demonstrates that the endogenous activation of MOR acts as a protective mechanism against intestinal inflammation.

MOR agonists prevent the development of colitis in T cell–restored SCID mice. To extend the previous observation to another murine model of chronic colitis, we investigated the effects of MOR agonists on T cell–mediated immune pathology in SCID mice reconstituted with CD4$^+$CD45RB^{hi} T cells. As shown in Figure 7a, the SCID mice restored with CD4$^+$CD45RB^{hi} T cells started to lose body weight 1 week after T cell transfer. At the end of the experiment, CD45RB^{hi} T cell–reconstituted SCID mice had an average body weight of 73.8% ± 3.4% of their initial weight, splenomegaly with a mean fold weight increase of 4.03 ± 0.53 compared with control mice, intestinal inflammation characterized by edema of the small intestine (Figure 7d), and diffuse thickening and shrinkage of the colon (Figure 7e), resulting in death in 40% of the mice (Figure 7b). Histologically, lesions scored 2 ± 0 were characterized by epithelial hyperplasia, goblet cell depletion, and a diffuse inflammatory infiltrate mainly located in the lamina propria (Figure 8, a and c). In contrast, none of the DALDA- or DAMGO-treated CD45RB^{hi} T cell–reconstituted SCID mice had splenomegaly (Figure 7d) or inflammatory changes in the intestine (score 0.2 ± 0.16) (Figure 8, a and d). The treated SCID mice gained weight throughout the course of the experiment without mortality and were indistinguishable from control mice reconstituted with CD45RB^{hi} and CD45RB^{lo} T cells (Figure 7, a and b).
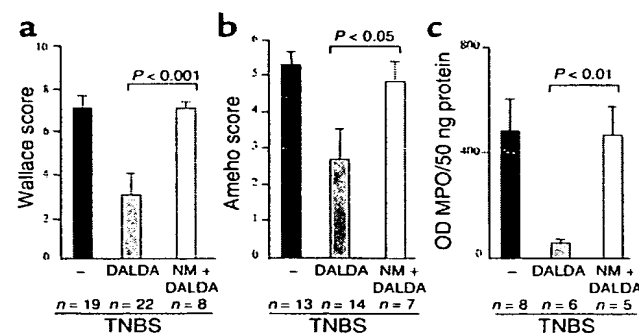
Figure 5
NM inhibits the anti-inflammatory effect of DALDA. Evaluation of (a) macroscopic and (b) histologic lesions and (c) quantification of MPO levels 4 days after TNBS administration in untreated mice (black bars) and mice receiving DALDA (10^{-2} mg/kg/d) (gray bars) or both NM and DALDA (white bars). The number of mice and statistical significance are indicated, and results are expressed as mean ± SEM.
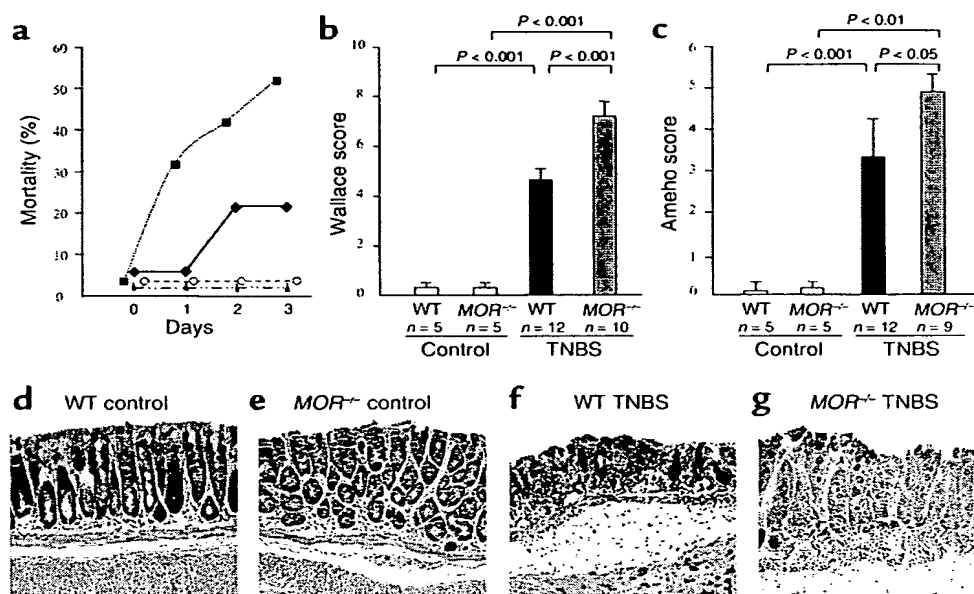
Figure 6

$MOR^{-/-}$ mice are more susceptible to TNBS-induced colitis. (a) Mortality rates of C57BL/6 wild-type and $MOR^{-/-}$ mice receiving vehicle (filled triangles and open circles, respectively) or TNBS (WT, filled diamonds; $MOR^{-/-}$, filled squares) daily for 3 days after TNBS administration. (b and c) Wallace macroscopic and Ameho histologic scores of C57BL/6 wild-type and $MOR^{-/-}$ mice 3 days after TNBS administration. The number of mice and statistical significance are indicated and results are expressed as mean ± SEM. (d–g) Histologic examination of colon sections of wild-type and $MOR^{-/-}$ mice receiving vehicle (d and e) or TNBS (f and g) (×250).

MOR agonist inhibits cytokine expression and T cell expansion. Although the above data performed in two different animal models of IBD underscore the importance of MOR activation in the regulation of colon inflammation, they do not address the possible mechanisms involved in this protective effect.

To evaluate the influence of MOR on the physiologic production of cytokines, we compared the expression of TNF-α, IL-4, and IFN-γ mRNA in the colon of mice receiving or not receiving the MOR agonist DALDA given once daily by subcutaneous administration for 8 consecutive days at a dosage of 10^{-2} mg/kg/d. As shown in Figure 9a, treatment with DALDA induced a significant inhibition of the basal production of TNF-α, IL-4, and IFN-γ mRNA in the colon. Conversely, administration of the MOR antagonist NM (2.5 mg/kg/d for 10 days) significantly increased the colon expression of these cytokines, indicating that a ligand-MOR interaction mediates the physiologic regulation of these cytokines in the colon (Figure 9a). To examine a direct relationship between MOR and cytokine expression in the colon, we compared the mRNA levels of cytokines in the colon of $MOR^{-/-}$ and wild-type mice. A two- to tenfold increase in the expression of TNF-α, IL-4, and IFN-γ mRNA levels was found in the colon of $MOR^{-/-}$ mice compared with controls (Figure 9b). Next, we investigated the roles of MOR agonist in the modulation of inflammatory cytokine expression in the colon of mice sacrificed 4 days after TNBS administration or 4 weeks after CD4⁺CD45RB^{hi} T cell reconstitution. Low

concentrations of TNF-α and IL-1β mRNA were present in the colon of control mice (Figure 9, c and d). After colitis induction, colon concentrations of TNF-α and IL-1β mRNA were significantly increased, reflecting a major inflammatory reaction both in TNBS-induced colitis and in T cell–restored SCID mice (Figure 9, c and d). In contrast, administration of MOR agonists normalized TNF-α and IL-1β mRNA concentrations in the colon in the two experimental models of colitis (Figure 9, c and d). Similarly, TNBS-induced colitis in $MOR^{-/-}$ mice was associated with increased expression of TNF-α (10.6 ± 1.1 TNF-α mRNA molecules per 10^4 β-actin molecules) and IL-1β (37.9 ± 20.3 IL-1β mRNA per 10^4 β-actin molecules) compared with wild-type mice with colitis (respectively, 4.1 ± 0.8 TNF-α mRNA per 10^4 β-actin molecules and 6.3 ± 3.5 IL-1β mRNA per 10^4 β-actin molecules, $P < 0.01$).

Since expansion of transferred T cells in the recipient SCID mice is also considered to play a crucial role in the pathogenesis of colitis (61), we compared the number of CD4⁺ T cells in the colon and spleen of CD45RB^{hi} T cell–reconstituted SCID mice treated or not treated with the MOR agonist DALDA (Table 1). As shown in Table 1, colitis was accompanied by an expansion of CD4⁺ T cells in the colon and spleen of untreated individual SCID mice. In DALDA-treated CD45RB^{hi} T cell–reconstituted SCID mice, colon tissues of five mice had to be pooled to obtain quantifiable numbers of CD4⁺ cells. Table 1 shows that treatment with DALDA significantly suppressed the expansion of CD4⁺ T cells in the colon
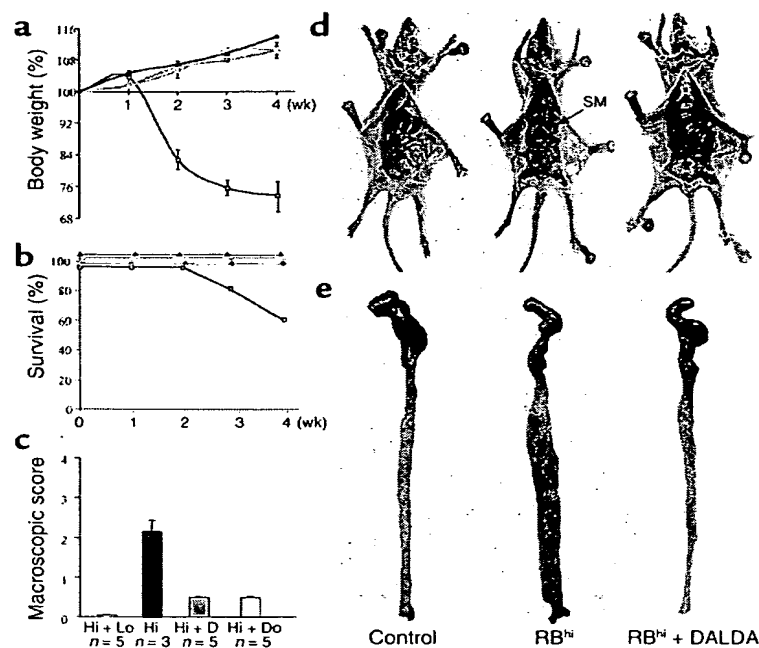
Figure 7

MOR agonists prevent wasting disease and colitis in SCID mice after T cell transfer. Weekly evaluation of change in weight (mean ± SEM) (**a**) and percent survival (**b**) in SCID mice during the 4 weeks after cell transfer. Untreated SCID mice after transfer of both $CD4^+CD45RB^{lo}$ and $CD45RB^{hi}$ T cells (diamonds) or after reconstitution with $CD4^+CD45RB^{hi}$ T cells (squares). SCID mice reconstituted with $CD4^+CD45RB^{hi}$ T cells and treated with DALDA (10^{-2} mg/kg/d) (triangles) or DAMGO (10^{-3} mg/kg/d) (circles). (**c**) Macroscopic scores (mean ± SEM) evaluated 4 weeks after T cell transfer in control SCID mice reconstituted with both $CD4^+CD45RB^{lo}$ and $CD45RB^{hi}$ T cells (Hi + Lo) and in SCID mice with colitis reconstituted with $CD4^+CD45RB^{hi}$ T cells without any treatment (Hi) or after subcutaneous administration of DALDA (10^{-2} mg/kg/d) (Hi + D) or DAMGO (10^{-3} mg/kg/d) (Hi + Do). (**d** and **e**) Representative macroscopic lesions in control SCID mice after transfer of $CD4^+CD45RB^{lo}$ and $CD45RB^{hi}$ (RB^{hi}) T cells (control) and in SCID mice with colitis after reconstitution with $CD4^+CD45RB^{hi}$ T cells without any treatment (RB^{hi}) or after subcutaneous administration of DALDA (10^{-2} mg/kg/d) (RB^{hi} + DALDA). SM, splenomegaly.

and spleen of $CD45RB^{hi}$ T cell–reconstituted SCID mice. Similar results were obtained when SCID mice were treated with DAMGO (data not shown).

Discussion

Many studies have documented the broad activities of MOR, particularly in the control of nociception (2), respiration (70, 71), cardiovascular functions (72), mood (73), and gastrointestinal secretion (3, 5). Recently, immunomodulatory activities of endogenous and exogenous μ-, κ-, and δ-opioid compounds have been described, but their in vivo effects during colon inflammation remain unknown (74). In view of the high level of MOR expression in the intestinal tract, particularly during inflammation, we have analyzed the effects of two selective MOR agonists, DALDA (49) and DAMGO

(75), on intestinal inflammation induced by TNBS in mice. In this well-described and validated experimental model of colitis (41), we showed that the two MOR agonists administered before or after colitis induction by TNBS are useful for the prevention of intestinal inflammation and also provide therapeutic benefit in established inflammatory lesions of the colon. Pretreatment with NM completely abolished the protective effects of DALDA and DAMGO on intestinal inflammation, suggesting that the activity of these agonists during colitis may result from an interaction with opioid receptors. Further, our results show that $MOR^{-/-}$ mice are more susceptible to TNBS-induced inflammation and suggest a tonic action of MOR in the protection against colon inflammation. To extend these results to another experimental model of colitis, DALDA or DAMGO

Figure 8

MOR agonists prevent histologic colitis in reconstituted SCID mice. (**a**) Histologic scores (mean ± SEM) evaluated 4 weeks after T cell transfer in control SCID mice reconstituted with both $CD4^+CD45RB^{lo}$ and $CD45RB^{hi}$ T cells (Hi + Lo) and in SCID mice with colitis reconstituted with $CD4^+CD45RB^{hi}$ T cells without any treatment (Hi) or after subcutaneous administration of DALDA (10^{-2} mg/kg/d) (Hi + D) or DAMGO (10^{-3} mg/kg/d) (Hi + Do). (**b–d**) Representative histologic sections of the colon in control SCID mice after $CD4^+CD45RB^{lo}$ and $CD45RB^{hi}$ T cell transfer (control) (**b**) and in SCID mice with colitis reconstituted with $CD4^+CD45RB^{hi}$ T cells without any treatment (RB^{hi}) (**c**) or after subcutaneous administration of DALDA (10^{-2} mg/kg/d) (RB^{hi} + DALDA) (**d**). ×250.
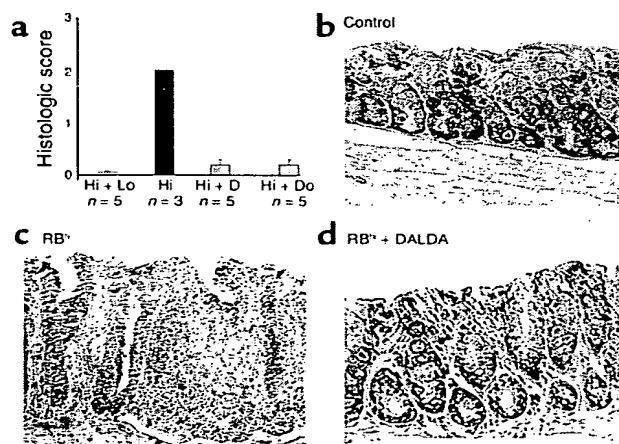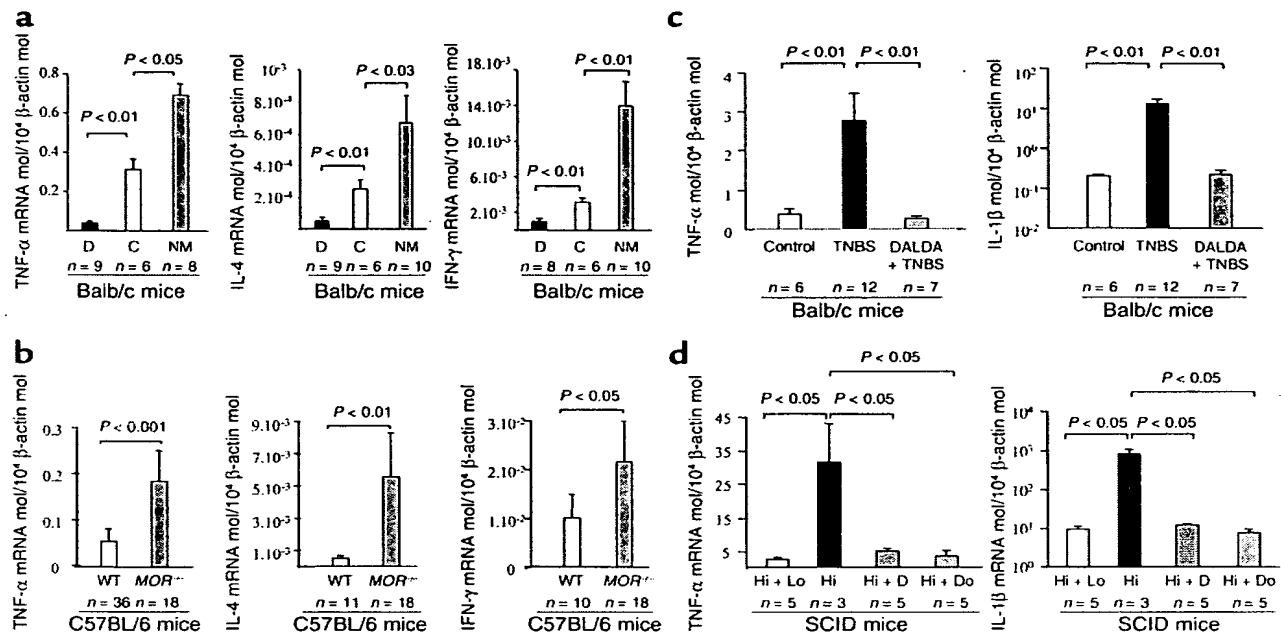
Figure 9

MOR regulates expression of inflammatory and immunoregulatory cytokines in the colon. (a and b) Quantification of TNF-α, IL-4, and IFN-γ mRNA concentrations in the healthy colon of (a) Balb/c mice receiving vehicle (C) for 8 days, the MOR agonist DALDA (D) for 8 days, or the opioid antagonist NM administered subcutaneously for 10 consecutive days and in colon of (b) adult $MOR^{-/-}$ mice and their wild-type littermates. (c) Quantification of inflammatory cytokine levels four days after treatment in the colon of Balb/c mice receiving vehicle only (Control), TNBS only, or DALDA followed 4 days later by TNBS. (d) Quantification of inflammatory cytokine levels in the colon 4 weeks after T cell transfer in control SCID mice reconstituted with both $CD4^+CD45RB^{lo}$ and $CD45RB^{hi}$ T cells (Hi + Lo) and in SCID mice with colitis reconstituted with $CD4^+CD45RB^{hi}$ T cells without any treatment (Hi) or after subcutaneous administration of DALDA (10^{-2} mg/kg/d) (Hi + D) or DAMGO (10^{-3} mg/kg/d) (Hi + Do). The number of mice and statistical significance are indicated and results are expressed as mean ± SEM.

was administered in SCID mice for 4 weeks after the transfer of $CD45RB^{hi}$ T cells. In this model of colitis dependent of T cell proliferation and activation, subcutaneous administration of MOR agonists effectively prevented colon inflammation, as evidenced by the absence of macroscopic and histologic lesions. Taken together, our studies with selective MOR agonists and with MOR knockout mice show that MOR might exert control of inflammatory responses in the intestine.

Numerous mechanisms may be proposed to explain the underlying anti-inflammatory effects of MOR during colitis. In the gut, the inhibitory effects of opioids on intestinal motility are believed to be mediated mainly by MOR located in the central nervous system (76, 77). However, since MOR is also expressed on various cells in peripheral tissues (11, 78–80), it may be speculated that there is a modulation of inflammation by peripheral MOR. In our study, the two selective MOR agonists, DALDA and DAMGO, and the MOR antagonist NM were selected for their inability to cross the blood-brain barrier (52–54). We therefore conclude that the protective effects of MOR during colon inflammation are probably mediated by peripheral and not central mechanisms.

Acute and chronic forms of colitis are considered to be mainly mediated by a dysregulation of inflammatory and immunoregulatory cytokine production (16, 17,

35, 81) and enhanced T cell proliferative responses (26, 27). In the present study, the TNBS-induced expression of TNF-α and IL-1β mRNA in the colon was completely abolished by treatment with MOR agonists in wild-type mice and significantly enhanced in $MOR^{-/-}$ mice compared with their littermates. To further determine whether MOR was directly responsible for the regulation of cytokine expression in the colon, we quantified cytokines in wild-type mice without colitis that were treated with MOR agonist and in $MOR^{-/-}$ mice. A 30–80% inhibition of the physiologic expression of TNF-α, IL-4, and IFN-γ mRNA in the healthy colon of mice was observed after DALDA administration and

Table 1
Number of $CD4^+CD45RB^{hi}$ T cells

	Spleen	Colon
$RB^{hi} + RB^{lo}$	0.52 ± 0.01	0.4 ± 0.02
RB^{hi}	12.5 ± 1.2	17.5 ± 2.6
$RB^{hi} + DALDA$	0.55 ± 0.05	0.4 ± 0.03

Splenocytes and mononuclear cells that had infiltrated the spleen and colon were collected, counted, and stained with TC-conjugated anti-CD4 and PE-conjugated anti-CD3 monoclonal antibodies. Percentages of total cells represented by different cell types were analyzed by cytofluorometry and the absolute number of $CD4^+$ T cells was calculated. Results are expressed as millions of cells per mouse.

with a two- to fourfold increase in cytokine levels with NM. Similar results were found in the colon of $MOR^{-/-}$ mice, which spontaneously expressed more inflammatory and immunoregulatory cytokines than did their wild-type littermates. Although these results suggest that the observed modifications of cytokine expression in the mouse colon are causally related to MOR activation, they do not identify which cell populations are responsible for the MOR-dependent effects.

Colitis in SCID mice arises from proliferation of transferred $CD4^+$ T cells initially in peripheral lymphoid organs such as the spleen, followed by accumulation of these cells in the intestine leading to Th1 cell–mediated chronic inflammation (82). In our study, administration of MOR agonists prevented the development of splenomegaly and inhibited the expansion of $CD4^+$ T cells in the spleen and colon of $CD45RB^{hi}$ T cell–reconstituted SCID mice. Based on these data, we propose that the anti-inflammatory effects of MOR in the colon are mediated at least in part by the regulation of cytokine expression and expansion of T cells, two important immunologic events required for development of colon inflammation in mice and patients with IBD (83).

In conclusion, our findings extend the understanding of the regulation of inflammation in the colon and stress that selective agonists of peripheral MOR have preventive and therapeutic intestinal anti-inflammatory effects. The property of these MOR agonists to selectively activate peripheral MOR would prevent adverse events occurring classically with morphinomimetic compounds such as euphoria, respiratory depression, sedation, nausea, and addiction (84). Moreover, the fact that MOR expression is particularly prominent in inflamed tissue compared with healthy tissue suggests that the treatment with MOR agonists may be clinically advantageous in patients with chronic inflammatory diseases characterized by multiple recurrences, such as IBD. The main therapeutic goals in patients with IBD are the control of intestinal inflammation and the treatment of the most common clinical features, i.e., abdominal pain and diarrhea. Since MOR agonists combine analgesic functions, inhibition of intestinal motility, and anti-inflammatory effects, we suggest that these compounds are promising therapeutic agents for patients with IBD.

Acknowledgments

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Mini-review

Opioids and opioid receptors in the enteric nervous system: from a problem in opioid analgesia to a possible new prokinetic therapy in humans[☆]

Peter Holzer[*]

Department of Experimental and Clinical Pharmacology, Medical University of Graz, Universitätsplatz 4, A-8010 Graz, Austria

Abstract

The gut is a neurological organ, which implies that many neuroactive drugs such as opioid analgesics can seriously disturb gastrointestinal function, because many of the transmitters and transmitter receptors present in the brain are also found in the enteric nervous system. One of the most common manifestations of opioid-induced bowel dysfunction is constipation which results from blockade of peristalsis and intestinal fluid secretion. The discovery of opioid receptor antagonists with a peripherally restricted site of action, such as N-methylnaltrexone and alvimopan, makes it possible to normalize bowel function in opiate-treated patients without compromising central opioid analgesia. There is emerging evidence that opioid receptor antagonists may also have prokinetic actions, reversing pathological states of gastrointestinal hypomotility that are due to overactivity of the enteric opioid system.
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Keywords: Alvimopan; Constipation; Enteric nervous system; Intestinal peristalsis; N-methylnaltrexone; Opioid-induced bowel dysfunction; Peripherally restricted opioid receptor antagonists; Prokinetic effects

Prologue. Although hardly containing any opiates, poppy seeds represent a delicacy of the Hungarian, Bohemian and Austrian cuisine, to name a few. It is not totally inappropriate, therefore, that endogenous opioid peptides were identified with a gastrointestinal (GI) bioassay in which opioid receptor agonists are quantified by their ability to inhibit electrically evoked contractions of the guinea-pig ileum. The explosion of neuropeptide research that followed the discovery of opioid peptides led Manfred Zimmermann to initiate the foundation of the European Neuropeptide Club and to be elected as its inaugural president. At the Second Meeting of the Club in 1992, Manfred Zimmermann gave a dinner speech in the Villa Le Molina near Pisa, in which he deliberated on the bidirectional brain-gut connection. He mentioned that motivations determining brain thought and action are likely to have a strong input from the gut, mediated by a wealth of neuropeptides and their receptors, and that the gut therefore may even have had political impact in history. This issue had previously been contemplated about by the French philosopher Voltaire who once remarked: "The fate of a nation has often depended on the good and bad digestion of its Prime Minister." Manfred Zimmermann went on to hypothesize

that, along the same neuronal network, the gastronomic richness we are enjoying now in a free Europe may facilitate progress in the unity of Europe.

The gut as a neurological organ. The function of the GI tract is not only to ensure the metabolic survival of the body but also to sort the ingested food in terms of its nutritive, toxic and pathogenic properties. These tasks are under multiple control systems among which neurons are particularly important. Since the alimentary canal is equipped with the largest collection of neurons outside the central nervous system (CNS), the gut can rightly be considered as a neurological organ [16]. The communication network of the enteric nervous system (ENS) involves acetylcholine, tachykinins (substance P, neurokinin A), nitric oxide, adenosine triphosphate, vasoactive intestinal polypeptide, opioid peptides, neuropeptide Y and 5-hydroxytryptamine as major transmitters. Enteric neurons supply all layers of the alimentary canal and thus are in a position to regulate virtually each aspect of digestion [16].

The ENS is arranged in polarized circuits that typically are composed of intrinsic primary afferent neurons, a variable number of interneurons and excitatory or inhibitory output neurons to the effector tissues (motor, secretomotor and vasodilator neurons). As the intrinsic primary afferent neurons supply the information that is required to regulate digestion according to need, the ENS issues its own programmes to

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govern the activity of the GI effectors independently of the CNS [16]. A typical ENS programme is the motor pattern of intestinal peristalsis which involves a temporally and spatially coordinated interaction of several polarized pathways of the ENS with the muscular layers of the intestine and thereby propels the intestinal contents in an aboral direction. GI ion and fluid secretion, circulation, endocrine and immune activity are also regulated by the ENS [16].

The gastrointestinal tract as a victim of neuroactive drugs. The significant role which neurons play in GI function has implications that extend beyond gastroenterology. On the one hand, various diseases and disorders of the GI tract are now thought to be related to neuropathies of enteric, sensory or autonomic neurons, alterations in gut-brain communication or changes in the brain-gut axis [16]. On the other hand, neuroactive drugs such as those used in neurology, psychiatry, anaesthesiology and intensive care medicine can disturb ENS function, because many of the transmitters and transmitter receptors present in the brain have also been localized to the ENS. This is particularly true for opioid receptors and adrenoceptors whose activation by opiates and catecholamines, respectively, interferes with ENS pathways involved in motility and secretion [6,7].

Adverse effects of opiates on gut function: opioid-induced bowel dysfunction. Morphine and related opioid analgesics are the mainstay of therapy in many patients with moderate to severe pain. A delay in GI transit and constipation are the most common and often disabling side effects of opiates, which result from blockade of propulsive peristalsis, inhibition of intestinal ion and fluid secretion and an increase in intestinal fluid absorption [6]. Importantly, though, constipation is just one symptom of an often under-recognized condition known as opioid-induced bowel dysfunction (OBD), whose manifestations also include incomplete evacuation, abdominal distension, bloating, abdominal discomfort and increased gastro-oesophageal reflux and which may lead to secondary complications such as pseudo-obstruction of the bowel, nausea, vomiting as well as interference with oral drug administration and absorption [10,20]. Although centrally mediated effects of opiates to slow GI transit have also been implicated in the pathophysiology of OBD, opiate-induced blockade of gut motility correlates better with opiate concentrations in the ENS than with opiate concentrations in the CNS [10]. It is, therefore, widely assumed that the adverse effects of opiates on GI function result primarily from interaction with opioid receptors in the gut [20].

Opioids and opioid receptors in the gastrointestinal tract. The adverse effect of opioid analgesics on GI function is consistent with the expression of opioid peptides [5,11] and opioid receptors [9,27] by distinct enteric neurons and intestinal muscle cells. When released from these neurons, opioid peptides are likely to play a transmitter role in the enteric regulation of propulsive motility and secretory processes [6,15,26]. The inhibitory effect of opioid receptor agonists on peristalsis is thought to arise primarily from interruption of transmission within enteric nerve pathways

governing muscle contraction [30]. Transmission is blocked via a presynaptic site of action, whereby the release of acetylcholine and other excitatory transmitters is attenuated, although postsynaptic effects have also been described [6]. However, the action of opiates on GI motility is subject to species differences. Thus, besides inhibiting peristalsis, opiates may block propulsive motility also by evoking tonic spasms in the intestine of humans and other species. The effect of opiates to contract intestinal muscle may involve depression of nitric oxide release from inhibitory enteric neurons [2,21] or direct activation of muscle cells that express opioid receptors [14,23]. Induction of stationary segmentations combines with inhibition of peristalsis and depression of secretory activity to bring about constipation.

The actions of opioids on the GI tract are mediated by multiple opioid receptors. Studies with isolated tissues from the human intestine suggest that delta-, kappa- and mu-opioid (OP_1, OP_2 and OP_3) receptors contribute to opiate-induced inhibition of muscle activity [1,3]. Peristalsis in the rat intestine is blocked by delta- and mu-opioid, but not kappa-opioid, receptor agonists [6], whereas peristalsis in the guinea-pig intestine is suppressed by activation of kappa- and mu-opioid, but not delta-opioid, receptors [26]. Opiate-induced inhibition of cholinergic transmission in the guinea-pig gut is likewise mediated by mu- and kappa-opioid receptors [18].

Management of opioid-induced bowel dysfunction by opioid receptor antagonists with a peripherally restricted site of action. The symptoms of OBD are predominantly mediated by peripheral mu-opioid receptors [10,20], given that mu-opioid receptors take a prominent position in mediating opiate actions in the gut of humans and other species and the most effective opioid analgesics are mu-opioid receptor-selective agonists. In view of the additional presence of delta- and kappa-opioid receptors in the human GI tract it appears unlikely that the GI adverse effect profile of opioid analgesics could be overcome by the use of delta- or kappa-opioid receptor-selective agonists. An alternative approach that has therefore been chosen is to selectively target peripheral opioid receptors with orally administered opioid receptor antagonists that have limited systemic absorption [10, 20]. As a number of studies has shown, this approach is successful in preventing OBD while saving the analgesic action of opiates which is preferentially mediated by central mu-opioid receptors. The search for gut-selective opioid receptor antagonists has been spurred by the discovery of the mu-opioid receptor-selective agonist loperamide which is used as an antidiarrhoeal drug without having an analgesic action.

The first attempt to selectively target opioid receptors in the periphery was made with the pan-opioid receptor antagonist naloxone and related tertiary opioid receptor antagonists such as nalmefene [10,17]. Since the systemic bioavailability of oral naloxone is as low as 2% because of extensive first-pass metabolism, this compound has been found to improve OBD without alleviating opiate-induced analgesia. However, the therapeutic index of naloxone turned out to be very narrow and

its utility limited because of the need to titrate peripherally versus centrally active doses [28].

A break-through advance was the development of quarternary opioid receptor antagonists such as N-methylnaltrexone whose absorption following oral administration is very low (oral bioavailability < 1%) and which does not cross the blood–brain barrier [10]. When this compound is given together with a centrally active opioid receptor agonist, the analgesic action of the opiate is maintained whereas the peripheral adverse effects on GI function are prevented in non-rodent animals and humans [10]. The species-dependence of the peripheral selectivity of N-methylnaltrexone is related to demethylation of the compound to naltrexone in rodents but not humans, naltrexone in turn being able to reach the CNS. Apart from its therapeutic potential, the action of N-methylnaltrexone to decrease the GI side effects of acute and chronic opiate treatment while preserving analgesia reinforces the concept that OBD is primarily brought about by activation of opioid receptors in the gut. Furthermore, the separation of the central wanted from the peripheral unwanted effects of opiates with N-methylnaltrexone allows for a more aggressive use of opioid analgesics with better pain relief but fewer side effects [10].

Following the proof of concept with N-methylnaltrexone (which is a non-selective, though mu-opioid receptor-preferring antagonist), a mu-opioid receptor-selective antagonist with a peripherally restricted site of action was developed. This compound, alvimopan (ADL 8-2698, formerly known as LY246736), has both low systemic absorption (oral bioavailability of 0.03% in dogs) and a limited ability to enter the CNS [25]. This spectrum of properties enables alvimopan to prevent morphine from delaying GI transit in healthy subjects without antagonizing central morphine effects such as analgesia and pupillary constriction [22]. A similar activity profile was seen in patients treated with opiates for chronic pain or opioid addiction, in which alvimopan reversed OBD without compromising opioid-induced analgesia or provoking CNS symptoms of opioid withdrawal [25]. Furthermore, alvimopan was found to improve the manage-

ment of postoperative ileus in patients who underwent abdominal surgery and received opioids for acute postoperative pain [29]. In this study, the time to achieve normal bowel function after the operation and the duration of hospitalization were shortened and the overall incidence of GI side effects including postoperative nausea and vomiting was reduced by alvimopan, whereas analgesia was not compromised [29].

Opioid receptor antagonists with a peripherally restricted site of action as possible prokinetic drugs. There is good reason to predict that opioid receptor antagonists with a peripherally restricted site of action may become important adjuncts of opioid analgesic therapy in order to avoid OBD [10,19,25]. I want to propose here that peripherally restricted opioid receptor antagonists may also have potential to alleviate intestinal motor stasis unrelated to opiate use. Such a prokinetic action can be envisaged from the finding that the pan-opioid receptor antagonist naloxone as well as selective mu- and kappa-opioid receptor antagonists can per se facilitate propulsive peristalsis in the guinea-pig isolated small intestine (Fig. 1) [6,15,26]. It follows that endogenous opioid peptides released in the course of propulsive motility [8] play an important role in the neural control of peristalsis as they dampen peristaltic performance via activation of mu- and kappa-opioid receptors [26]. This inference is consistent with the effect of mu- and kappa-opioid receptor antagonists to enhance the release of acetylcholine from the myenteric plexus [4] and of naloxone to increase the release of substance P during peristalsis [8]. Similarly, N-methylnaltrexone is able to enhance neurogenic contractions in the isolated human small intestine [31].

Another reason to conjure a prokinetic action of opioid receptor antagonists derives from the ability of naloxone to rescue propulsive motility from blockade by noradrenaline, atropine and hexamethonium [13,15]. It thus seems as if motor inhibition caused by activation of adrenoceptors, muscarinic and nicotinic acetylcholine receptors in the ENS involves endogenous opioids which block distinct enteric transmission processes [15]. If it can be proved that

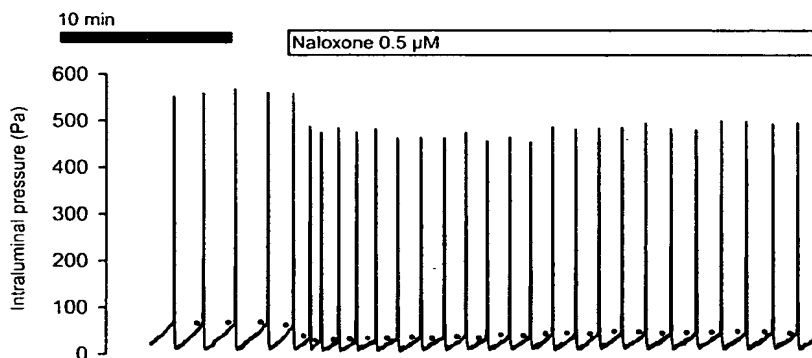Fig. 1. Stimulant effect of naloxone (concentration as indicated) on propulsive peristalsis in an isolated segment of the guinea-pig small intestine. The stimulant effect of naloxone is evident from a decrease in the peristaltic pressure threshold at which peristaltic waves are elicited (indicated by dots) and from an increase in the frequency of peristaltic waves. For details see Shahbazian et al. [26].

upregulation and/or overactivity of the opioid system in the ENS is causally involved in pathological states of GI motor inhibition, there would be a rationale to test the prokinetic potential of peripherally restricted opioid receptor antagonists in various GI hypomotility states. There is indeed preliminary evidence that naloxone has beneficial effects in idiopathic chronic obstipation [19], intestinal pseudo-obstruction [24] and constipation-predominant irritable bowel syndrome [12].

Acknowledgements

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TODAY
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Pharmacotherapy of alcohol dependence: Targeting a complex disorder

George A. Kenna[1,2]

[1]Center for Alcohol and Addiction Studies, Brown University, Providence, RI 02912, USA
[2]Clinical Pharmacist, Kent County Memorial Hospital, Warwick, RI 02912, USA

Consistent replication of drug studies in heterogeneous populations of alcohol-dependent individuals has been disappointing, although more is being learned about differential drug response by subtypes of alcoholics. Because alcohol affects multiple neurotransmitters, new strategies that focus on drugs with complex mechanisms of action or drug combinations is the trend in this field. Finding clinically efficacious drugs or drug combinations that benefit broad or specific subpopulations of alcoholics, drives current trials.

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Les Iversen – Department of Pharmacology, University of Oxford, UK

Introduction

Alcohol abuse and dependence are widespread as an estimated 14 million American adults abused alcohol or were dependent on it in 1992 and approximately 10% of Americans will be affected by alcohol dependence sometime during their lives [1]. Alcohol dependence, characterized by the preoccupation with alcohol use, tolerance and withdrawal, is a chronic disorder with genetic, psychosocial and environmental factors influencing its development and manifestations [2]. Among other neurochemicals, studies demonstrate the significance of β-endorphin, dopamine (DA), serotonin (5-HT), γ-amino butyric acid (GABA) and glutamate for the development and maintenance of alcohol dependence [3] (Table 1). To date most pharmacotherapy trials have focused on single pharmacological agents

(Table 2). However, because of the failure to find consistent results with these drug therapies, investigating the efficacy of combining drugs that target multiple neurotransmitter systems or genes [4], is perhaps more important to the development of future pharmacotherapies for alcohol dependence treatment. This review highlights the most promising drugs and drug combinations that are in various phases of human trials.

Present pharmacotherapies for alcohol dependence

Despite the number of studies performed in this area, few drugs for alcohol dependence are approved in the U.S. Disulfiram is an irreversible inhibitor of aldehyde dehydrogenase leading to increased levels of acetaldehyde, a toxic intermediate in alcohol metabolism. Patients who take disulfiram and drink alcohol experience an increased dilation of arterial and capillary tone producing hypotension, nausea, vomiting, flushing, headache and possibly in some, worse. In one trial, no significant difference in abstinence rates between groups taking placebo, 1 mg or 250 mg of disulfiram was found, and demonstrated that when individuals were compliant, regardless of group, alcohol consumption was reduced [5]. An independent review of the data suggest the efficacy for disulfiram is fair as clinical trials determine disulfiram has inconsistent therapeutic value, but most effective in patients

E-mail address: G.A. Kenna (George_Kenna@Brown.edu)

Table 1. Overview of major neurotransmitters, functions and alcohol-related behaviors

Neurotransmitter	General function	Specific action by alcohol	Alcohol-related function
Dopamine (DA)	Regulates motivation, reinforcement and fine motor control	Initiates a release at the nucleus accumbens (NA) either directly or from projections via the mesolimbic system from the ventral tegmental area (VTA).	Mediates motivation and reinforcement of alcohol consumption.
Serotonin 5-HT	Regulates bodily rhythms, appetite, sexual behavior, emotional states, sleep, attention and motivation	The brain 5-HT system can modulate alcohol intake by two different mechanisms: modulation of the dopamine-mediated reinforcing properties of alcohol via $5\text{-}HT_2$ and $5\text{-}HT_3$ receptors; and suppression of alcohol intake by activation of $5\text{-}HT_{1A}$ receptors.	Might influence alcohol consumption, intoxication and development of tolerance through $5\text{-}HT_1$ receptors; might contribute to withdrawal symptoms and reinforcement through $5\text{-}HT_2$ receptors; and might modulate dopamine release through $5\text{-}HT_3$ receptors, thereby increasing alcohol's rewarding effects.
γ-Aminobutyric acid (GABA)	Serves as the primary inhibitory neurotransmitter in the brain	Causes tonic inhibition of dopaminergic projections to the VTA and NA. Prolonged alcohol use causes a down-regulation of these receptors and a potential for decreased inhibitory neurotransmission.	Might contribute to intoxication and sedation; inhibition of GABA function following drinking cessation can contribute to acute withdrawal symptoms.
Glutamate	Serves as the major excitatory neurotransmitter in the brain	Alcohol inhibits excitatory neurotransmission by inhibiting both N-methyl-D-aspartate (NMDA) and non-NMDA (kainite and AMPA) receptors. Up-regulation of these receptors to compensate for alcohol's antagonistic effect occurs after prolonged exposure to alcohol resulting in an increase in neuroexcitation.	Might contribute to acute withdrawal symptoms; inhibition of glutamate function following drinking cessation; might contribute to intoxication and sedation.
Opioid peptides	Regulates various functions as well as produce morphine-like effects, including pain relief and mood elevation	Alcohol stimulates β-endorphin release in both the NA and VTA area. β-Endorphin pathways can lead to increased DA release in the nucleus accumbens via two mechanisms. (1) β-Endorphins can disinhibit the tonic inhibition of GABA neurons on DA cells in the VTA area. This leads to a release of DA in the NA area. (2) β-Endorphins can stimulate DA in the NA directly. Both mechanisms could be important for alcohol reward.	Contributes to reinforcement of alcohol consumption, possibly through interaction with dopamine.

Adapted from Swift ([3] p. 209).

who are highly motivated, adherent with its use or use is supervised [6].

Naltrexone's principal site of action is at the μ receptors in the mesolimbic pathway which putatively blocks the reinforcing effects of alcohol by decreasing DA release in the nucleus accumbens (NAc). Studies using naltrexone report the opioid antagonist is more effective than placebo in reducing craving [7,8], heavy drinking and increasing the percentage of non-drinking days, but does not necessarily enhance abstinence [6]. Although these studies support the efficacy of naltrexone, others report limited utility for the drug only when individuals were highly compliant [9] or even not at all [10]. Correlates of effective naltrexone therapy include compliance, a family history of alcoholism, more reported somatic distress and craving of alcohol on intake to treatment, and lower educational status. Although not yet approved, a once a month naltrexone formulation using Medisorb® technology is due to be released in 2005. In a recently ended Phase III

trial, patients treated with Vivitrex® 380 mg experienced a statistically significant reduction in the rate of heavy drinking relative to placebo, however, women reported no significant difference.

Acamprosate, a structural analogue of GABA and available in Europe for 20 years, was recently approved by the Food and Drug Administration (FDA) in the U.S., to promote abstinence in recently detoxified individuals. Although the exact mechanism of action is unknown, the drug is thought to restore glutamatergic-mediated inhibitory and excitatory neurotransmission in the NAc. In studies involving more than 4000 participants, the majority of acamprosate studies report positive effects over placebo for abstinence, relapse and percentage of non-drinking days [6,11,12]. As a result of these data, acamprosate was released in the U.S. January 2005. Despite the important contributions these drugs make to alcohol treatment, abstinence or even reduced heavy drinking levels still remain elusive for many. This suggests the need for

Table 2. Selected double-blind placebo controlled trials for alcohol dependence (completed)

Medication (dose)	Type of agent	Weeks/study design	Alcoholic subtype	Drinking/non-drinking days	Craving	Relapse	References
Disulfiram (1 mg, 250 mg)	Aversive	52/double-blind placebo controlled	AD	+	NR	NR	Fuller et al. [5]
Naltrexone (50 mg)	Opiate antagonist	12/double-blind placebo controlled	AD	+	+/0	+	O'Malley et al. [7]
Naltrexone (50 mg)	Opiate antagonist	12/double-blind placebo controlled	AD	+	+	+	Volpicelli, et al. [8]
Acamprosate	NMDA antagonist	48/double-blind placebo controlled	AD	+	0	+	Sass et al. [11]
Acamprosate (1332 mg or 1998 mg)	NMDA antagonist	24/double-blind placebo controlled	AD	+	+	+	Pelc et al. [12]
Fluoxetine (60 mg)	SSRI	12/double-blind placebo controlled	AD/Type A	0	NR	0	Kranzler et al. [14]
			AD/Type B	−	NR	0	
Fluoxetine	SSRI	12/double-blind placebo controlled	AD/MD	+	NR	NR	Cornelius et al. [15]
Sertraline (200 mg)	SSRI	14/double-blind placebo controlled	AD/Type A	+	NR	0	Pettinatti et al. [16]
			AD/Type B	0	NR	0	
Ondansetron (4 µg/kg)	5-HT3 antagonist	12/double-blind placebo controlled	Early-onset	+	NR	NR	Johnson et al. [20]
			Late-onset	0	NR	NR	.
Topiramate (up to 300 mg)	Mixed-action	12/double-blind placebo controlled	AD	+	+	NR	Johnson et al. [33]

NR indicates data not reported; (+) medication significant compared to placebo ($P < .05$); (−) significant difference favoring placebo; 0, no significant difference; (±) interaction of subgroup or trend favoring medication; AD, alcohol-dependent; GAD, generalized anxiety disorder; MD, major depression; Type A, later onset of alcohol-related problems, severe dependence, fewer childhood risk factors, alcohol-related problems and psychopathological dysfunction; Type B, early-onset of alcohol-related problems, increased number of childhood risk factors, family history of alcoholism, greater severity of dependence; early-onset, onset of alcohol problems <25 years; late-onset, onset of alcohol problems >25 years.

discovering medications providing more efficacious treatments.

Targeting neurotransmitters
Serotonergics
Serotonin (5-HT) dysfunction probably contributes to the development of alcoholism [13]. Serotonin's receptors contribute to alcohol use in animals, as alcohol increases basal levels of 5-HT affecting receptors. Of the seven distinct families of 5-HT receptors, three are known to contribute to alcohol dependence: $5-HT_{1A}$ receptors might be associated with alcohol consumption and the development of tolerance, $5-HT_2$ receptors with reward and $5-HT_3$ receptors with the development of reinforcement. Based on such evidence, several serotonergic drugs have been examined, but with inconsistent results [13]. Presently only sertraline and ondansetron appear to show any promise with certain subtypes of alcoholic patients and fluoxetine with depressed alcoholics.

Despite reductions in drinking in lab studies with animals and in human drinking sessions in which subjects have been administered SSRIs, most double-blind placebo-controlled studies using SSRIs have not reduced drinking or any other measures of alcohol dependency. Recent research however, suggests that because of the heterogeneity of the disease, perhaps subtypes of alcoholics respond differently to SSRIs. For example, although found to be effective for reducing alcohol consumption in depressed alcoholics [14], fluoxetine 60 mg was no better than placebo in reducing alcohol consumption in alcohol-dependent patients. In post hoc analyses, however, two alcohol subtypes were found to respond differentially to the medication: The Type A subtype, relatively uncomplicated in their alcohol use history, did not respond at all to fluoxetine, and the Type B subtype with an earlier onset of alcoholism, severe alcohol dependence, more childhood risk factors and psychopathology actually increased their alcohol consumption [15]. In a follow-up study that pre-stratified patients by subtype, alcohol-dependent patients were administered 200 mg a day of sertraline or placebo [16]. This study confirmed that Type B alcoholics treated with sertraline reported more drinking days compared with those taking placebo but conflicted with previously reported results, and demonstrated that sertraline treatment in Type A alcoholics to be associated with fewer drinking days and a greater likelihood of sustained abstinence. A follow-up study using sertraline in alcohol-dependent patients stratified

by their age of onset of alcoholism will hopefully provide some clarity to these data (see Table 3).

Animal studies demonstrate that the 5-HT$_3$ receptor facilitates some of the biochemical and behavioral effects of alcohol through midbrain DA release. 5-HT$_3$ antagonists are consistently shown to suppress alcohol preference in animal studies [17], with recent evidence suggesting the 5-HT$_{3A}$ receptor subunit requisite for 5-HT$_3$ antagonist-induced reductions in alcohol consumption [18].

Ondansetron, a 5-HT$_3$ receptor antagonist, has functionally opposite affects to SSRIs and blocks serotonin agonism at the 5-HT$_3$ receptor. Studies demonstrate that ondansetron can be effective for early-onset (EOA) but not late-onset alcoholics (LOA) where age of onset of alcoholism (younger versus older than 25 years old), is the basis for subtyping alcoholics [19]. In a placebo-controlled trial, 271 participants were stratified into EOA and LOA subtypes by 1, 4 and 16 μg/kg twice daily doses of ondansetron compared to placebo [20]. Patients with EOA who received ondansetron showed significant reductions in drinking (particularly those receiving 4 μg/kg twice daily) compared to LOA's across all groups. In sum, ondansetron continues to be examined for individuals with early-onset alcoholism (Table 3).

The reasons for these differential effects are unknown, however, one hypothesis suggests that alcoholics with a biological predisposition have a dysregulation of serotonergic function primarily associated with serotonin transporter (SERT) function [19]. The polymorphic variation of the SERT (the 5'-HTTLPR) is hypothesized to be involved with the effectiveness of ondansetron and sertraline in EOA and LOA alcohol-dependent individuals, respectively. Given that epidemiologic studies demonstrate alcohol dependence has an approximately 50–60% heritability [21] the prospect for positive outcomes to drug therapy at least partly dependent on genetic predisposition in some alcoholics is strong. Recent studies have therefore attempted to delineate the genetic components associated with alcohol dependence [4,22–25]. These findings highlight the important role that 5-HT plays in alcohol consumption, although drug trials using serotonergics have had difficulty delineating responders from non-responders.

Neuroleptics

Animal studies suggest that fluctuating DA levels contribute to craving leading in turn to relapse in abstinent alcoholics [26,27]. Strategies aimed at up-regulation of D2 receptor (DRD2) levels in the NAc, which might be significantly reduced in alcoholics, could be particularly beneficial during continued abstinence of alcohol [28]. Dopamine regulation in general, and in particular DA antagonism might be an important target for drug development. Reward associated with alcohol cues manipulating DA release by the mesolimbic pathway and positive symptoms of schizophrenia seem to share similar dopaminergic dysfunction [29]. Neuroleptics that regulate DA occupancy at DRD2, possibly causing an up-regulation of DRD2, might be associated with reduced positive symptoms of schizophrenia and reduced substance use [30].

Haloperidol, tiapride, olanzapine and clozapine [27] have all demonstrated various degrees of efficacy reducing craving, alcohol consumption or increasing abstinence. Although theoretically interesting drugs to study, the risks associated with the side effects of typical or atypical neuroleptics have outweighed the benefits for using DA antagonists as serious treatments for alcoholism.

Aripiprazole, an atypical neuroleptic, has few of the limiting side effects associated with these related medications. Aripiprazole is a partial dopamine agonist (PDA) with mixed HT$_{1A/2A}$ activity. As with other PDAs, aripiprazole has a high affinity to bind to DA receptors but with low intrinsic activity, subsequently acting as an antagonist or agonist under conditions of hyper- or hypodopaminergic availability, respectively. Additionally as a mixed HT$_{1A/2A}$ receptor drug, aripiprazole independently shows significant effects reducing alcohol use in both animals and humans [27].

Unlike the previously noted neuroleptics used in alcohol treatment studies, aripiprazole causes few extrapyramidal side effects, lacks strong histamine activity suspected of causing sedation, causes relatively little elevation in prolactin levels or QT$_c$ prolongation, has no propensity for weight gain, and the risk of agranulocytosis is no greater than for the typical neuroleptics [27]. Because of its potential as a treatment for alcohol dependence, Bristol-Myers Squibb is funding a double-blind placebo controlled trial using APZ in recently abstinent alcohol-dependent patients that should complete recruiting in 2005. Although the use of neuroleptics by alcoholics can signify an important step into future pharmacotherapeutic approaches, direct dopaminergic manipulation obviously does not represent the entire solution to alcoholism treatment.

Antiepileptics

Midbrain and cortical DA pathways mediate alcohol's rewarding effects. Alcohol consumption increases GABA receptor activity which inhibits midbrain DA neurons and facilitates DA neurotransmission. Non-N-methyl-D-aspartate (NMDA) glutamate antagonists oppose GABA activity, thereby decreasing DA release. Topiramate and gabapentin are FDA-approved antiepileptics. Topiramate is thought to have multiple mechanisms of action, including enhanced GABA$_A$ inhibition which results in decreased dopamine facilitation in the midbrain, antagonism of kainate to activate the kainite or AMPA type glutamate receptor subtypes, and inhibition of carbonic anhydrase Type II and IV isoenzymes [31]. Gabapentin reduces glutamate and increases GABA neurotransmission in the brain [32]. Theoretically therefore, the unique

Table 3. Comparison of selected drug trials for alcoholism

	Pros	Cons	Latest developments	Who is working on this strategy?	References
Sertraline 200 mg/day	Selectively targeting LOA subtypes	Most probably little treatment benefit for EOA subtype	N = 160; 80 EOA and LOA; project ends 31 May 2008	Kranzler, H., University of Connecticut	[14,16]
Ondansetron 0.5 mg/day and naltrexone 100 mg/day	Examining potential mechanisms of action in subpopulations	Not a clinical trial	Projects end 31 May 2008	Rohsenow, D., Brown University	[7,8,20,37]
Topiramate 300 mg (maximum dose)	Potentially mimics actions of alcohol without the reinforcement	Not tested in recently abstinent alcoholics; optimal dose unknown	N = 368; 6-week titration, 8-week maintenance and about 7 days taper; ends in 2005	Ortho McNeil Pharmaceutical	[31,33]
Ondansetron 4 μg/kg twice a day	Treatment matching in EOA based on 5'-HTTLPR might result; nominal side effects	Most probably little treatment benefit for LOA subtype	N = 320, 160 with LL SERT and 160 with the SS/SL SERT; project ends 4/30/05	Johnson, B.A., University of Texas	[19,20]
Ondansetron 4 μg/kg twice a day and Topiramate 300 mg (maximum dose)	The combination of ondansetron and topiramate might be additive among EOA	Ondansetron dosing by weight does not easily translate to clinical practice	N = 360, project ends 8/31/09	Johnson, B.A., University of Virginia	[19,20,31,33]
Aripiprazole 30 mg	Multiple mechanisms of action	No pre-clinical or clinical data; not tested in actively drinking alcoholics; optimal dose unknown	N = 266, 12 weeks; also receive weekly psychotherapy; end 2005	Bristol Myers Squibb	[27]
Naltrexone and acamprosate (COMBINE)	Targeting positive and negative reinforcement	Not tested in actively drinking alcoholics	Pharmacotherapy phase is ended; results not reported	Multicenter	[36]
SR141716, rimonabant	Unique mechanism of action; potentially useful for nicotine cessation and weight loss	Little research on drugs that block the endocannabinoid system, that is, potential side effects	N = 40 Lab study of non-treatment seeking volunteers using cannabinoid-1 antagonist	David. G., NIAAA	
Naltrexone and sertraline	Combination might yield better abstinence rates	Both drugs have gastrointestinal side effects	198 Alaskan Native Americans; project ends 8/31/05	O'Malley, S., Yale University	[7,8,16]
Aripiprazole and topiramate	Combining two drugs with multiple mechanisms of action can have a synergistic effect	Expense of treatment might be prohibitive in clinical practice	N = 20 in non-treatment seeking volunteers; project ends in 2005	Kenna, G.A., Brown University	[27,33]
Gabapentin as an adjunct to naltrexone for alcoholism	Practical application to clinical practice		Project ends 8/31/07; examine if alcoholics receiving naltrexone and adjunctive gabapentin will have less relapse than those treated with naltrexone alone	Anton, R., Medical University of South Carolina	[7,8,32]

Abbreviations: EOA, early-onset alcoholism; LOA, late-onset alcoholism; NIAAA, National Institute for Alcohol Abuse and Alcoholism.

pharmacology of these medications is well suited to the treatment of alcohol dependence or withdrawal and could normalize the brain dysregulation seen during the early abstinence period.

In a double-blind placebo-controlled trial, 150 men and women were titrated up to a maximum of 300 mg of topiramate per day during a 12-week period [33]. Participants in the topiramate arm reported significantly fewer drinks per day and per drinking days, significantly fewer drinking days, significantly more days of abstinence, and significantly less craving than placebo. Because abstinence was not a goal at the start of the study, the medication might be more beneficial during the abstinence-initiation phase of treatment [33]. Although there was no differential subtype effect, co-administration with other medications such as ondansetron (Table 3) or other drugs, might produce enhanced efficacy outcomes particularly for the specific subtypes of alcoholic patients. Although gabapentin has seen increased use as an alternative to benzodiazepines in alcohol withdrawal syndrome [34], its use as a potential adjunct to naltrexone for promoting abstinence in alcoholism is also being investigated (Table 3).

Multidrug therapies

There is a growing trend toward multiple drug study designs (Table 3). Recent interest in combining therapeutic agents for the treatment of alcoholism is based on the suggestion that derangement of multiple-neurotransmitter systems is likely to underlie biological predisposition to the disease. Thus, combining medications targeted at different neurotransmitters should produce an added or synergistic clinical response.

The basis for combining naltrexone and acamprosate lies in positive and negative reinforcement of alcohol dependence [35]. Naltrexone can influence positive reinforcement of alcohol use affected by the β-endorphin opiate system which modulates dopamine release. Negative reinforcement, which occurs when one drinks to reduce anxiety, or relieve withdrawal, might be helped by the abstinence reinforcing effects of acamprosate. Although each drug individually appears to provide modest yet significant effects on treatment and drinking outcomes, taking advantage of naltrexone's reduction in relapse rates and acamprosate's reduced drinking frequency and abstinence promotion was the basis for the COMBINE trial which combined both in addition to behavioral strategies for alcohol dependence [36]. The results of that study will be released during the spring of 2005 and should provide invaluable data to elucidate the usefulness of these pharmacological treatments both alone and together in addition to psychological interventions.

Naltrexone has been administered with ondansetron in EOA patients. In an 8-week, double-blind, placebo-controlled trial, the combination was found to significantly reduce drinks per day and per drinking day and had a positive effect

on the percentage of days abstinent compared to placebo [37]. The authors suggest that adding ondansetron to naltrexone can provide a synergistic action in the EOA patient subtype. Further study examining potential mechanisms of action are currently being explored.

The co-administration of topiramate and ondansetron is also under study. As noted, ondansetron has a differential effect among EOA but not LOA, presumably by ameliorating an underlying serotonergic dysfunction associated with EOA. Additionally topiramate was shown to be superior to placebo at improving the drinking outcomes for alcohol-dependent patients regardless of subtype. Combining the two medications can result in a more efficacious pharmacotherapy particularly for early-onset alcoholics.

Aripiprazole can target fluctuating dopamine levels and serotonin receptors facilitating continued abstinence [27]. Topiramate blocks AMPA receptors and behaviorally targets abstinence initiation [33]. Potentially combining these drugs could be synergistic and a safety and tolerability study examining the effects of aripiprazole (20 mg and 30 mg) and topiramate (200 mg and 300 mg) both alone and together with alcohol is currently being performed.

Summary and conclusions

After years of research into understanding the biological mechanisms underlying alcohol consumption, momentum for finding more effective pharmacotherapies for alcoholism finally appears to be escalating with many more agents in development not presented in this review. Some large pharmaceutical manufacturers and academic institutions have several medications and compounds at various stages of pre-clinical or clinical development for alcohol dependence. Although there are no guarantees that newer compounds will be more efficacious than current pharmacotherapies, the involvement of major corporations provides a new source of research excitement and capital expenditure to the field. Nonetheless much work remains to be performed and many questions about pharmacotherapy of alcoholism remain (see Outstanding issues).

As so much research in this area is contradictory or unreplicated, speculation as to which direction the field is heading is futile. Almost certainly though, genetics will play a greater role in the future. Of newly available drugs, it is difficult to predict if acamprosate or injectable naltrexone will match the same level of disuse of oral naltrexone and disulfiram. Sertraline and ondansetron can have potential for specific subtypes of alcoholics, but many questions remain surrounding how a clinician can best define who will respond to which treatment and at what dose. Of drugs in clinical trials by pharmaceutical manufacturers, topiramate is probably closest to FDA submission and appears to have a clinical effect across a broad population of alcoholics regardless of subtype, yet many questions remain about optimal dosing and length of

treatment. Aripiprazole, although strong on pharmacological rationale, is without any known pre-clinical or pilot data testing its effectiveness.

Recognizing the complexity of alcoholism, treatments are becoming more multifaceted. This approach can provide more effective treatments in controlled trials, yet has its own set of drawbacks such as compounded chance of side effects, complex dosage regimens leading to adherence problems, additional cost to many who are without prescription insurance or otherwise limited financial means, and sufficient knowledge transfer to physician and non-physician practitioners.

As a result of advances in the understanding of the neural mechanisms of alcohol dependence, investigating medications affecting multiple neuronal systems reducing craving or blocking the reinforcing affects of alcohol although minimizing side effects, are important goals for researchers and subsequent transfer to clinicians in the field. Multidrug protocols have great potential for treating alcoholism. Although many studies are currently determining the clinical efficacy of such an approach, acceptance of pharmacotherapy by patients and treatment providers might in the end be the key to realizing the full potential any drug or combination can have.

Outstanding issues

- Could combining serotonergic drugs (SSRI and 5-HT$_3$) be synergistic?
- Is topiramate effective in recently abstinent alcoholics?
- Would a dose higher than 300 mg of topiramate be effective for treatment resistant alcoholics?
- Would combining multiple drugs to target specific aspects of the addiction cycle be more effective than single drug approaches, for example, combining naltrexone and acamprosate, ondansetron and topiramate or aripiprazole and topiramate?
- Does increasing the binding to μ-opioid receptors (over that of naltrexone) lead to a greater reduction in alcohol consumption?
- What role will pharmacogenomics play in targeting drug development for alcoholism?
- Are there other undiscovered alcohol subtypes?

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Alvimopan, a selective peripherally acting μ-opioid antagonist*

M. CAMILLERI

Clinical Enteric Neuroscience Translational and Epidemiological Research (CENTER) Program, Mayo Clinic College of Medicine, Rochester, MN, USA

Abstract *Alvimopan is a novel, peripherally acting μ-opioid antagonist that is being developed for the management of acute postoperative ileus and for the reversal of the delayed gastrointestinal and colonic transit that result in symptoms such as constipation, nausea and motility disorders in patients treated with opiate analgesics. There is a clinical need for effective medications for the treatment of postoperative ileus and opiate-induced constipation and other motility disorders. This review addresses the basic and applied pharmacology and current evidence for the use of the medication, alvimopan, in clinical gastroenterology.*

Keywords *constipation, ileus, irritable bowel, gastrointestinal transit.*

INTRODUCTION

This review addresses the pharmacology (basic and applied) and current evidence for the use of the medication, alvimopan, in clinical gastroenterology. The biological rationale for the use of a μ-opioid antagonist is based on increased understanding of the role of opiate receptors in the peripheral modulation of gut functions, and the new insights in the pathophysiology of diseases such as postoperative ileus. Endogenous opiates alter gut functions and this is easily understood when one considers the dramatic changes in gut function caused by commonly used μ-opioid agonists such as codeine or morphine. Modulation of the function of enkephalinases, which

normally inactivate endogenous opiates is also effective therapeutically as acetorphan, an enkephalinase inhibitor, is used in the treatment of acute diarrhoea.[1, 2] This article focuses on the μ-opioid antagonist, alvimopan, which represents a novel orally active medication to restore the balance in situations when endogenous or exogenous opiates alter gut homeostasis, e.g. by immunomodulatory or neuromodulatory functions.

OPIATE RECEPTORS

Though there are five types of opiate receptors with biological actions in gut motor and sensory functions, studies in humans have focused on the roles of three specific opiate receptors: μ, δ and κ. The distribution of μ receptors differs from that of κ receptors; there is a greater abundance of μ receptors in all layers of the rat intestine submucosal regions and the interstitial cell layer.[3] In contrast, there were more κ than μ receptors in the myenteric plexus. Using a combination of mRNA quantification and immunohistochemical visualization, stomach and proximal colon had the largest expression of both μ and κ opiate receptors.[4] μ-, δ- and κ-opioid receptors mediate the effects of endogenous-opioids and opiate drugs. Recently, Sternini *et al.*[5] examined the distribution of μ-opioid receptors in the guinea-pig and human gastrointestinal tract in relation to endogenous ligands, to functionally distinct structures in the gut, and to δ- and κ-opioid receptors. In the guinea-pig, μ-opioid receptor immunoreactivity is confined mainly to the myenteric plexus. μ-opioid receptors in myenteric neurons are most numerous in the small intestine, followed by the stomach and the proximal colon. μ-opioid receptors couple to activation of a potassium channel, inhibition of calcium channels, and reduced production of cAMP leading to inhibition of neurotransmitter release.[6] Immunoreactivity studies show that μ-opioid receptors are dense in the muscle layer and the deep muscular

plexus, where they are in close proximity with the interstitial cells of Cajal. This distribution closely matches the pattern of enkephalin. Enteric neurons having μ-opioid receptors comprise functionally distinct populations of neurons of the ascending and descending pathways of the peristaltic reflex. In human gut, μ-opioid receptor immunoreactivity is localized to myenteric and submucosal neurons and to immune cells of the lamina propria. In contrast, in guinea-pig, δ-opioid receptor immunoreactivity is located in both myenteric and submucosal plexuses, where it is predominantly in varicose fibres in the plexuses, muscle and mucosa.[5] κ-opioid receptor immunoreactivity appears to be confined to the myenteric plexus and to bundles of fibres in the muscle of guinea-pig.

μ-opioid receptors undergo endocytosis in a concentration-dependent manner, *in vitro* and *in vivo*. Pronounced endocytosis was observed in neurons from animals that underwent abdominal surgery and the authors suggest that this may play a role in the development of postoperative motor disturbances.[5]

μ-opioid receptors: role in peripheral modulation of gut sensory and motor functions

Recent reports have addressed the potential role of μ-opioid receptors in the peripheral control of gut sensory and motor functions.

Sensory function Grundy et al.[7] examined the sensitivity of mesenteric afferents supplying the rat small intestine to μ-opioid receptor ligands. Mesenteric afferent discharge was recorded electrophysiologically in response to [D-Ala^2, N–Me–Phe^4, Gly^5-ol]-enkephalin (DAMGO; 100 μg kg^{-1} i.v.), before and after treatment with the μ receptor antagonist, alvimopan (1 mg kg^{-1} i.v.). DAMGO markedly stimulated whole nerve mesenteric afferent discharge, an effect completely blocked by alvimopan. The response of mesenteric afferents to 2-methyl-5-hydroxytryptamine (30 μg kg^{-1} i.v.), bradykinin (0.1–1 μg kg^{-1} i.a.) and both low- and high-threshold distension (0–60 mmHg) was unaffected by alvimopan, suggesting that the effect of the μ-opioid receptor antagonist was relatively specific. In chronically vagotomized animals, the low-threshold response to distension was attenuated while the remaining high-threshold response was unaffected by alvimopan. As mesenteric afferent fibres are markedly stimulated by μ-opioid receptor agonists and this effect is blocked by alvimopan, the data suggest that alvimopan may influence the gastrointestinal reflex and behavioural responses to opiate treatment. How-

ever, alvimopan did not influence the normal sensitivity of intestinal afferents to chemical and mechanical stimuli that activate different subpopulations of vagal and spinal afferents.

Motor function In a summary of the published literature on experimental animal studies, naloxone and the peripherally acting μ-opioid receptor antagonists, alvimopan and methylnaltrexone, reversed morphine-induced inhibition of gastrointestinal transit in mice and rats and morphine- or loperamide-induced inhibition of castor oil-induced diarrhoea in mice.[8] The site of action of these agents differs and this influences the potentially beneficial analgesic effects of the opiate. Thus, at doses producing maximal reversal of morphine-induced effects upon gastrointestinal transit, only the central nervous system penetrant antagonist, naloxone, also reversed morphine-induced analgesia.[8] Both central and peripheral μ-opioid antagonists may affect gastrointestinal functions and/or visceromotor sensitivity in the absence of exogenous opioid analgesics.[8-10] These data suggest there is a constitutive role for endogenous opioid peptides in the control of gut physiology. Furthermore, in contrast to naloxone, alvimopan did not produce hypersensitivity to the visceromotor response induced by nociceptive levels of colorectal distension in a rodent model of postinflammatory colonic hypersensitivity. These data suggested that, in the periphery, endogenous μ-opioid receptor-mediated mechanisms do not regulate colonic sensitivity. The data support the hypothesis that peripherally acting μ-opioid antagonists may be able to selectively block μ-opioid receptors in the gastrointestinal tract, thereby preserving normal gastrointestinal physiology while not blocking the effects of endogenous opioid peptides or exogenous opioid analgesics in the central nervous system. These findings also suggested that μ-opioid agonists have a primary effect to inhibit the function of the gut in the periphery, whereas analgesic activity resides primarily in the central nervous system.[8]

Holzer's group also showed that the μ-opioid receptor antagonist, naloxone, stimulated peristalsis in the isolated guinea-pig small intestine, leading Holzer to propose that peripherally restricted μ-opiate receptor antagonists may also have potential to relieve intestinal stasis unrelated to opiate use.[11] *In vivo* studies performed in our laboratory in healthy humans confirm the potential for the peripherally restricted μ-opioid receptor antagonist, alvimopan, to stimulate colonic transit.

Overall, the recent data suggest that alvimopan may be useful for the treatment of gastrointestinal sequelae

following opiate treatment for postoperative or chronic pain.

In vivo effects of μ-opiates on gastrointestinal sensation and motility in humans

Sensory function Antinociceptive effects of μ-opioids are a mainstay in the management of somatic as well as visceral pain. The potential for the use of opiates in functional bowel diseases has also been tested with rectal distension in irritable bowel syndrome patients: i.v. infusion of fentanyl results in reduced sensation scores, but there is clearly a central effect of this agent as approximately 70% of the patients participating in the study identified the medication to which they were allocated and higher mental functions were also significantly affected.[12] It is still unclear whether a peripheral μ-opiate action on sensation can be achieved without central effects. In contrast, the κ-opioid agonist, asimadoline, is associated with greater maximum tolerated volume of a nutrient liquid meal, suggesting it alters gastric sensation, given the lack of effect on gastric volumes and reduction in sensory ratings at low, not at high (more noxious) distension pressures applied to the descending colon in healthy human volunteers.[13]

Motor function Effects of the different classes of opioid agonists on human gut motility are heterogeneous. Thus, Phillips' group[14, 15] showed that i.v. morphine induced MMC-like activity in the small bowel and variable effects on contractility of the colon. Kaufman et al.[16] showed that the effects of opiates resulted in retardation of gastrointestinal transit. Similarly, loperamide, a peripheral μ-opioid receptor agonist, was shown to inhibit jejunal motility and intestinal and colonic transit and to stimulate resting anal sphincter pressure.[17–19]

Others[19] evaluated the effects of synthetic recombinant beta-endorphin and identified not only induction of MMC activity but also activation of pyloric tonic and phasic pressure activity. Interestingly, these experiments also showed that high doses of peripherally-administered naloxone also resulted in stimulation of duodenojejunal phasic pressure activity[20], though the significance of these observations was not clearly identified during those studies performed 20 years ago.

More recently, Delgado-Aros et al.[13] showed the κ-agonist, asimadoline, had no significant effects on gastrointestinal transit, but it relaxed colonic and gastric tone during fasting and reduced sensation during non-noxious range stimuli applied to the stomach or colon.

GUT DISEASES ASSOCIATED WITH OPIATE MECHANISMS OR TREATMENTS

μ-opioids are beneficial in acute and chronic diarrhoea; loperamide is also used extensively in the control of diarrhoea and incontinence or urgency associated with irritable bowel syndrome.[17] The μ-opioid antagonists, naloxone and analogs such as methylnaltrexone, have been tested in small studies of irritable bowel syndrome[21] or opiate bowel dysfunction; they appear to be more promising in opiate bowel dysfunction. The demonstration of opiate receptors on enteric neurons, enkephalin production in enteroendocrine cells and the clinical efficacy of enkephalinases such as racedotril in the treatment of diarrhoea[1, 2] suggest that endogenous enkephalins may also influence gut motor and sensory functions.

Two syndromes associated with use of opiates used as analgesics are postoperative ileus (in which opiates are one of a large number of potential mechanisms leading to ileus) and opiate bowel dysfunction. Novel approaches are needed to provide pain relief without inducing such bowel dysfunction.

Postoperative ileus

Postoperative ileus (POI) is a universal period of cessation of intestinal function, typically occurring after abdominal surgery.[22] Proposed mechanisms include actuation of spinal and local sympathetic neural reflexes, inflammatory mediation and exacerbation by anaesthetic or surgical procedures.[23] There is a paucity of controlled data on the prevention and management of POI. Current management includes the use of postoperative care plans with early ambulation and diet, epidural anaesthesia and laparoscopic approaches for intestinal resection.[22,23]

Some procedures or agents have shown clinical benefit and these include use of laparoscopic surgery, thoracic epidurals, non-steroidal anti-inflammatory drugs, and opiate antagonists. Other procedures may be helpful with low risk of adverse effects. These include early feeding and ambulation, laxatives and possibly neostigmine. Table 1 summarizes the current understanding of the mechanisms and treatment of postoperative ileus.[23] Given the association with the use of opiate analgesia, the use of selective opiate antagonists has been proposed.[24]

A recent review evaluated the evidence for three main mechanisms, which are considered to be involved in the causation of POI, namely neurogenic, inflammatory and pharmacological mechanisms.[25] In the acute postoperative phase, mainly spinal and

Table 1 Summary of postoperative ileus [from Behm and Stollman (2003)[23]]

Pathophysiology: Mechanisms
  Spinal and local sympathetic neural reflexes
  Local and systemic inflammatory mediators
  Exacerbating factors
    Opioid analgesics
    Intraperitoneal surgery
    Degree of bowel manipulation
    Open surgical procedures
    Hypokalemia
Treatment options
  (A) Non-pharmacological
    Nasogastric tube decompression
    Early enteral nutrition
    Sham feeding
    Early mobilization
    Laparoscopic surgery
    Psychological preoperative preparation
  (B) Pharmacological
    Metoclopramide, cisapride, erythromycin,
    Opiate antagonists
    Epidural anaesthesia
    NSAIDs, COX-2 selective medications, laxatives
    Anti-adrenergic agents
    Cholinergic agents
    Neostigmine
    Multimodality therapy

supraspinal adrenergic and non-adrenergic pathways are activated. Recent studies, however, show that the prolonged phase of postoperative ileus is caused by an enteric inflammatory response and the subsequent recruitment of leucocytes into the muscularis layers of the intestinal segments manipulated during surgery. This inflammation impairs local neuromuscular function and activates neurogenic inhibitory pathways, which result in the inhibition of motility of the entire gastrointestinal tract. The mechanisms underlying the recruitment of the inflammatory cells, and their interaction with the intestinal afferent innervation, are increasingly understood. Opioids administered for postoperative pain control also contribute importantly to the reduction in propulsive gastrointestinal motility observed after abdominal surgery. However, there is increasing evidence of the potential immunomodulatory effects of opiates.

Opioids are known to alter immune function through numerous mechanisms by binding to specific-opioid receptors on leucocytes.[26–29] In general, μ-opioid agonists are reported to be primarily immunosuppressive, while δ-opioid agonists appear to be immuno-enhancing.[26,28] In POI, there is an important role of iNOS-derived NO within the monocyte/macrophage-inflamed intestinal muscularis[30]; opioids also

potentiate iNOS induction and NO release from phagocytes.[27] Thus, this aspect of opioid modulation of the immune system could be potentially harmful, and blockade of this response might be beneficial. At present, the optimal approach is unclear. A more thorough demonstration of the role of central *vs* peripheral mechanisms in opioid-induced immunosuppression[31] would also enhance understanding of the potential immunomodulatory role of alvimopan.

Opioids have also been shown to increase NO release from endothelial cells, which would theoretically decrease leucocyte adhesion. Such an opioid-modulated decrease in leucocyte recruitment could be advantageous in the postoperatively inflamed intestine.[32] However, as indicated above, some of these responses tend to be opioid receptor selective, and further experimentation is needed to delineate fully the individual roles of each receptor type.

Opioid-induced constipation and opioid-induced bowel dysfunction

Opioid-induced constipation is a common clinical problem for which there is currently no specific therapy.[33–36] Opioid-induced bowel dysfunction encompasses the symptom spectrum that reflects changes in upper and lower gastrointestinal function, e.g. anorexia, nausea, bloating, abdominal distension, as well as reduced stool frequency. Recent literature from relatively small studies suggests that a novel μ-opiate antagonist, such as methylnaltrexone, may be effective.[37,38] The efficacy of alvimopan in the reversal of the effects of opiates in opiate-induced constipation and postoperative ileus is discussed below.

THE μ-OPIOID ANTAGONIST, ALVIMOPAN

Chemistry and pharmacokinetics

Alvimopan is a white to light beige powder and is a single isomer; its chemistry is summarized in Fig. 1 and Table 2.[39,40] In *animals*, alvimopan has extremely
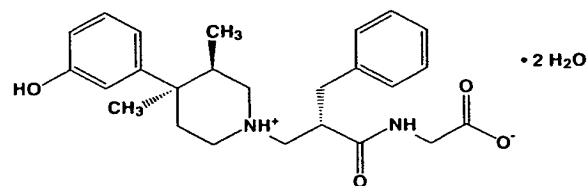
Figure 1 Chemical structure of alvimopan.

Table 2 Physical and chemical characteristics of alvimopan

Molecular weight	460.6
Molecular formula	$C_{25}H_{32}N_2O_4 \cdot 2H_2O$
pK_a	3.30, 8.70, 11.43 (extrapolated using methanol as co-solvent)
Melting point	210–213 °C
Specific rotation	51.79 ° at 589 nm, 163.00 ° at 365 nm (10 mg mL^{-1} in dimethyl sulfoxide)

low oral bioavailability and a short half-life in the systemic circulation, with a $t_{1/2}$ of approximately 10 min after i.v. dosing in dogs and rabbits. Intravenously or orally dosed drug stays mostly in the gastrointestinal tract, and there is no drug accumulation with chronic dosing of alvimopan.

In *humans*, the pharmacokinetic profile also is characterized by limited oral bioavailability (approximately 6%), which results in the predominance of the drug effects on the gut itself. A metabolite of alvimopan has been detected and is believed to occur via gut microfloral metabolism and not by hepatic metabolism. There is no evidence of cythochrome P450 metabolism or of glucuronide or sulphate conjugation of alvimopan. The major route of excretion is fecal, and the minor route of excretion is in urine. After oral administration, there is limited systemic absorption with an estimated $C_{max} = 14$ ng mL^{-1}; $t_{max} = 1.5$–3.0 h; and $t_{1/2} = 1.3$ h after oral administration of 12 mg dose.

Pharmacology

Alvimopan has high affinity for opioid receptors ($K_i = 0.77$, 40, and 4.4 nmol L^{-1} for μ, κ and δ receptors, respectively). It is a potent μ-opioid receptor antagonist following parenteral and oral administration and it distributes selectively (>200-fold selectivity) to peripheral receptors.[40–42]

Pharmacological data support the selectivity of alvimopan for the μ-opioid receptor *in vitro*, and its peripheral selectivity *in vivo*. Alvimopan is a selective and competitive antagonist at the μ-opioid receptor. In animal models of gastrointestinal function, alvimopan is a long-acting and potent antagonist of morphine-induced inhibition of gastrointestinal transit and of morphine- or loperamide-induced inhibition of castor oil-induced diarrhoea when administered orally to mice. Alvimopan selectively antagonizes the peripheral effects of morphine in physically dependent mice and has a negligible propensity to antagonize the

centrally-mediated analgesia produced by morphine when administered by either the i.v. or oral routes. The amide hydrolysis product of alvimopan, ADL 08-0011, may contribute to the potency and duration of alvimopan in models of gastrointestinal function.

Pharmacodynamic studies in humans

Alvimopan reversed the inhibition of orocecal transit induced by i.v. morphine (Fig. 2) and the effects of oral loperamide on colonic transit[43] (Fig. 3) without reversing pupillary constriction. Alvimopan also reversed MS Contin® inhibition of colonic transit ($P < 0.01$),[44] increased stool weight ($P < 0.05$), and did not reverse pupillary constriction or morphine-induced analgesia.[45,46] Recent studies using scintigraphy with a delayed-release capsule that delivers radiolabelled
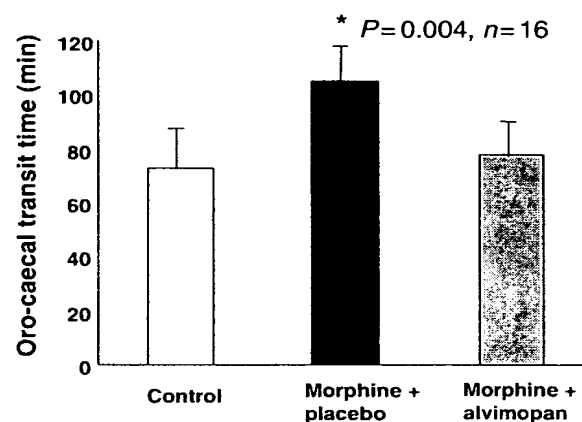
Figure 2 Alvimopan reverses morphine-induced delay in orocecal transit (data plotted from Liu et al.[45]).
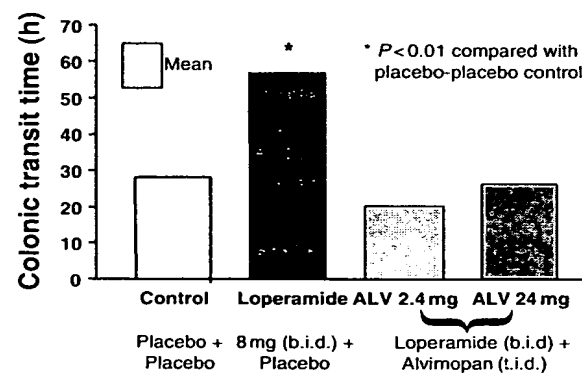
Figure 3 Alvimopan reverses loperamide-induced delay in colonic transit (data plotted from Callahan et al.[43]).

activated charcoal to the colon and a radiolabelled egg meal have shown that alvimopan, 12 mg b.i.d., accelerated colon transit and reversed high dose (30 mg q.i.d.) codeine-induced colonic transit delay in healthy volunteers (J. Gonenne, M. Camilleri and A.R. Zinsmeister, Mayo Clinic, Rochester, MN, USA, pers. comm.). This was also associated with normalization of codeine-induced retardation of small bowel transit and acceleration of ascending colon emptying (J. Gonenne, M. Camilleri and A.R. Zinsmeister, Mayo Clinic, Rochester, MN, USA, pers. comm.). Alvimopan acceleration of colonic transit in healthy volunteers not exposed to opiates is consistent with a prokinetic effect of this peripherally-acting μ-opiate antagonist. This observation suggests a physiological role of μ-opioid receptors in myenteric neurons in several species[5] and validates the prediction of Holzer[11] based on the pharmacological effects of naloxone in a peristaltic model of the isolated guinea-pig intestine.

Efficacy of alvimopan in opiate bowel dysfunction

μ-opiate antagonists have shown dose-related efficacy in the treatment of opiate bowel dysfunction. The efficacy of alvimopan has been tested in a phase II study of 75 individuals: alvimopan dose-dependently increased the number of bowel movements and stool weight and reduced hard stools and the need to strain severely to have bowel movements (Fig. 4).[39] Alvimopan also accelerated the time to first bowel movement in chronic pain patients receiving opiates.[47–50] This reversal of opiate-induced bowel dysfunction is achieved without inhibition of the pain-relieving effect of the opiate[45,46], and the efficacious dose in

patients exposed to chronic opiate medication is lower[49] than in people not previously exposed to opiates.

Efficacy of alvimopan in postoperative ileus

Two large phase III studies have now been published demonstrating efficacy of alvimopan in the management of postoperative ileus. Taguchi et al.[51] tested the hypothesis that the peripherally-acting μ-opioid antagonist, alvimopan, speeds recovery from postoperative ileus (Fig. 5) without antagonizing opioid analgesia. Wolff et al.[52] evaluated 510 patients scheduled for bowel resection or radical hysterectomy who were randomized (1 : 1 : 1) to receive alvimopan 6 mg, alvimopan 12 mg, or placebo orally 2 h or more before surgery, then twice a day (b.i.d.) until hospital discharge or for up to 7 days. The modified intent-to-treat population included 469 patients (451 bowel resection and 18 radical hysterectomy patients). Time to recovery of gastrointestinal function was accelerated for the alvimopan 6 mg [hazard ratio (HR) = 1.28; $P < 0.05$] and alvimopan 12 mg (HR = 1.54; $P < 0.001$) groups with a mean difference of 15 and 22 h, respectively, compared with placebo (Fig. 6). The time to hospital discharge order written was also accelerated in the alvimopan 12-mg group (HR = 1.42; $P = 0.003$) with a mean difference of 20 h compared with placebo. The incidence of adverse events was similar in all treatment groups, with the most common adverse effects being nausea, vomiting and abdominal distension. Thus, alvimopan accelerated gastrointestinal recovery and time to hospital discharge order written compared with placebo in patients undergoing laparotomy and was well tolerated.
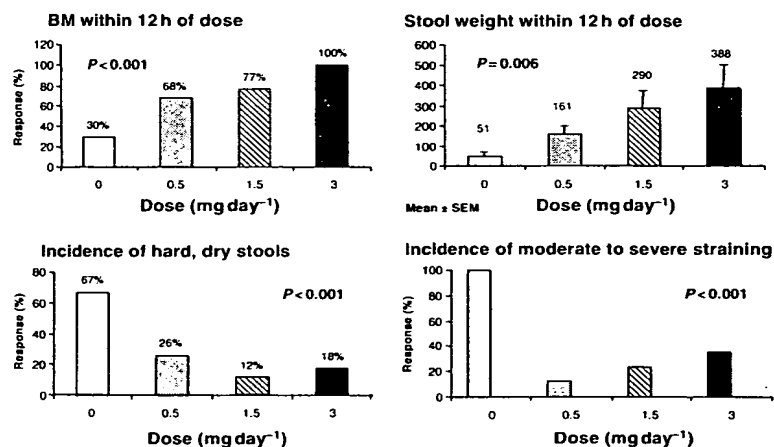
Figure 4 Effect of alvimopan in chronic-opioid bowel dysfunction, $n = 75$; alvimopan dose-dependently increases proportion with bowel movement, stool weight and consistency. Note increased straining, which may be related to increased frequency of bowel movements (data plotted from Schmidt[39]).

**Mean time to first bowel
Movement
reduced by 2.0 days**

Placebo							
1 mg							
6 mg						$P = 0.0132$	
	0	1	2	3	4	5	6

Days ± SEM

**Mean time to solid diet
reduced by 1.3 days**

Placebo							
1 mg							
6 mg						$P = 0.0001$	
	0	1	2	3	4	5	6

Days ± SEM

**Mean time to patient discharge
reduced by 1.4 days**

Placebo							
1 mg							
6 mg					$P = 0.0001$		
	0	1	2	3	4	5	6

Days ± SEM
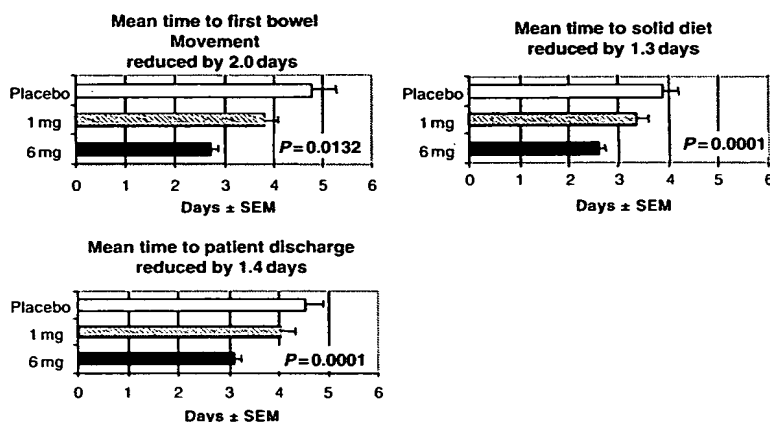
Figure 5 Effect of alvimopan and placebo on endpoints associated with postoperative ileus (reproduced from Taguchi et al.[51]).
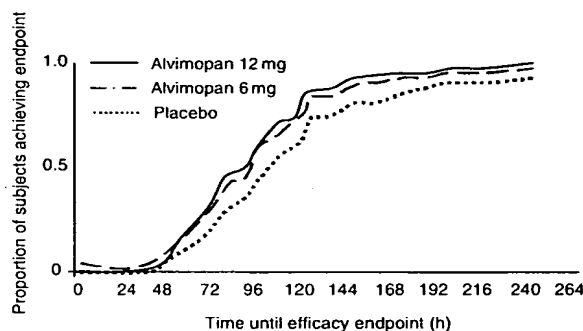
Figure 6 Comparison of effect of alvimopan and placebo in postoperative ileus using Cox proportional hazards model: 6 mg vs placebo, hazards ratio 1.28 ($P < 0.05$); 12 mg vs placebo, hazards ratio 1.54 ($P < 0.001$). Data reproduced from Wolff et al.[52].

Effects of alvimopan in patients with chronic constipation

Alvimopan accelerates colonic transit in healthy subjects[43,44] and this was confirmed recently by state-of-the-art scintigraphic studies which also demonstrated acceleration of ascending colon emptying (J. Gonenne, M. Camilleri and A.R. Zinsmeister, Mayo Clinic, Rochester, MN, USA, pers. comm.). These data suggest that the medication may also be an effective colonic prokinetic. The more rapid emptying of the ascending colon is typically associated with a looser stool consistency or increased stool weight[53] and results in the effective treatment of chronic constipation or constipation-predominant irritable bowel syndrome, as demonstrated with the 5-HT$_4$ agonist, tegaserod.[54, 55] The clinical relevance of these observations is confirmed by preliminary data suggesting that alvimopan shortens whole bowel transit time in

patients with chronic constipation.[56] Clinical efficacy data are awaited.

Safety of alvimopan in animals and humans

Nine studies were conducted to evaluate the effects of alvimopan on a variety of general pharmacological parameters in animals. These studies were designed to detect acute cardiovascular effects in conscious rats, conscious dogs, anaesthetized dogs, action potential effects in isolated Purkinje fibres, pulmonary effects in anaesthetized guinea pigs, acute behavioural changes in mice, renal effects in rats, acute effects on gastrointestinal motility in mice, and agonist/antagonist activity on isolated guinea-pig ileum. Alvimopan was inactive in these studies at doses up to 30 times greater than those demonstrating effects on reversal of-opioid-induced changes in GI motility. Based on these studies, alvimopan was determined to have a low potential for producing any general pharmacological effects in humans.

In humans, alvimopan has been studied in multiple Phase I and Phase II studies and over 1525 subjects have participated in Phase III studies. The longest continuous administration of alvimopan was in μ-opiate bowel dysfunction studies with a maximum treatment period of 23 days, and the highest dose of 120 mg was administered in a Phase I trial. Adverse events in all the studies conducted to date are mostly consistent with the pharmacological action of alvimopan in the gastrointestinal tract and with the subject population (healthy volunteers and subjects with μ-opioid-induced bowel dysfunction or postoperative ileus) under study. The most frequently reported adverse events were nausea, vomiting and abdominal discomfort. In chronic μ-opioid-induced bowel dysfunction, subjects were observed to be more sensitive

to the effects of alvimopan. Thus, the 3-mg dose of alvimopan was associated with an increased incidence of expected gastrointestinal adverse events, suggesting that the optimal dose of the antagonist in patients whose receptors are upregulated requires further study. No signs of central-opioid withdrawal, or reversal of analgesia were observed in these subjects.

Evaluation of the QT and-QTc interval on electrocardiograms has revealed no clinically significant prolongations related to alvimopan and there are no data to suggest cardiotoxicity in several hundred healthy participants or patients treated to date.

CONCLUSION

Alvimopan is an orally-active, peripherally-restricted μ-opioid antagonist that has promising prokinetic properties and is capable of reversing the effects of μ-opioids on delayed gastrointestinal transit. It represents a new class of compounds that is likely to impact significantly on therapeutics in such indications as postoperative ileus, μ-opioid bowel dysfunction and gastrointestinal motility disorders.

ACKNOWLEDGMENTS

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Pain, immunity, opiate and opioid compounds and health

George B. Stefano[1], Gregory L. Fricchione[2], Yannick Goumon[3], Tobias Esch[4]

[1] Neuroscience Research Institute, State University of New York, College at Old Westbury, Old Westbury, NY, U.S.A.
[2] Department of Psychiatry, Division of Psychiatry and Medicine, Massachusetts General Hospital, Boston, MA, U.S.A.
[3] Inserm, 575 "Physiopathology of the Nervous System", Strasbourg, France
[4] Charité – University Medicine Berlin, Institute for General Practice and Family Medicine, Berlin, Germany

Summary

We surmise that opioid peptides, i.e., methionine enkephalin, first arose during evolution as modulators of cellular immune function given their immune actions and the presence of enkelytin, a potent antibacterial peptide, and its precursor proenkephalin in animals 500 million years divergent in evolution. Pain probably emerged from this perspective because of its association with proinflammatory events. Endogenous morphine appears to exert positive effects on homeostasis by limiting the degree of excitation. Supporting this view is the fact that the mu3 opiate receptor subtype, which is opioid peptide insensitive and morphine selective, is coupled to constitutive nitric oxide release, which also has this down regulating action in neural, immune, vascular and gastrointestinal tissues. Thus, morphine down regulates immune processes in addiction, an action/function that it appears to normally perform when the situation calls for this action and by so doing in this natural setting, sustains life.

key words: morphine • pain • amygdala • nitric oxide • μ3 opiate receptor • opioid peptides • methionine enkephalin • enkelytin

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Author's address: Dr. G.B. Stefano, Neuroscience Research Institute, State University of New York, College at Old Westbury, Old Westbury, NY 11568-0210, U.S.A., e-mail: gstefano@sunynri.org

BACKGROUND

Over hundreds of millions of years living organisms evolved numerous and diverse strategies to protect themselves against threats, i.e., microbes, harsh environments and the trauma associated with rapidly changing circumstances. These environmental challenges are usually referred to as perturbations if weak and 'stress' if stronger, and protective mechanisms to overcome stressful situations include the so called "stress response" [1,2]. Those successful strategies gave such endowed organisms the ability to pass on these processes, including those that could be considered health promoting. Thus, long living organisms today retain many successful strategies for survival, enabling them to live a relatively long life in reasonably good health. Probably the most advantageous coping strategy that evolved recently is cognition [3], enabling man to effect this process directly, speeding up the evolvement of even more protective strategies. As with any process, too much "thinking" can lead to inactivity and by so doing becomes detrimental [4,5]. Taken together, we surmise that our physiological processes contain health-promoting information, which we have only begun to understand. In this review, we examine only opioid and opiate chemical messengers for their health promoting actions in diverse organisms. This is important since opiate alkaloids are primarily identified for their negative role in addiction and related activities.

OPIOID PEPTIDES

Opioid pentapeptides, i.e., methionine enkephalin, stimulate cytokine release and immunocyte chemotaxis and induce conformational changes in immunocytes indicative of activation [6]. These activities are phylogenetically ancient since they also occur in invertebrates, animals that are 500 million years divergent in evolution from mammals [6,7]. Invertebrates contain a mammalian-like proenkephalin molecule [7], which contains the potent antibacterial peptide enkelytin [7] that exhibits a 98% sequence identity with mammalian enkelytin [7]. The presence of enkelytin strengthens the association of opioid peptides with immune-related activities.

Since these chemical messengers are present in both invertebrates and man, in the past we demonstrated that opioid peptides stimulate immunocyte chemotaxis and phagocytosis as well as the secretion of cytokines, and the simultaneously liberated enkelytin would attack bacteria immediately (Figures 1,2) [8]. This process would allow time for the immunocyte-stimulating capabilities (i.e., recruitment) of the opioid peptides to manifest themselves. Interestingly, this same scenario may occur in neural tissues, i.e., via microglia. In neural tissues, proenkephalin processing may also lead to enkelytin 'liberation', suggesting that neurons may exhibit bactericidal innate immune functions. We surmise these mechanisms have evolved to supplement immune actions by covering the latency period before total or partial immune activation occurs. The same phenomenon, e.g., simultaneous increase in opioid peptides and enkelytin, is found in human plasma in patients undergoing coronary artery bypass grafting [9]. Taken together, it can be argued that the co-processing and liberation of enkelytin and [Met]enkephalin represents a unified neuroimmune protective response to an immediate threat to the organism, regardless of what form the stimulation takes. Such a unified response might thus represent an important survival strategy since it promotes health [8].

Pain

The concept of pain [10] has proved a difficult issue to approach. Nevertheless, there is a wealth of literature documenting the ability of opioid peptides and opiate alkaloids in ameliorating the sensation of pain [11,12]. In this regard, why, then, is an antibacterial peptide found within proenkephalin containing methionine enkephalin, a naturally occurring analgesic inducing molecule, and why has this association endured for at least 500 million years? The close association of enkelytin and opioid peptides, i.e., methionine enkephalin, probably reflects the fact that both types of molecules have evolved to fight the presence of microbes. Bacteria and viruses are persistent factors in the environment and are a threat to any organism, regardless of the evolutionary time period. Thus, in order to survive and reproduce, organisms had to evolve processes to combat this immediate non-cognitive threat. The association of enkelytin and opioid peptides probably represents such a successful strategy [8].

Pain and immune function

Once the association between enkelytin and opioid peptides was established in evolutionary terms, organisms needed an early detection/surveillance system by which they could continuously monitor microbial penetration and growth. What better alerting process than one that signaled attention by creating a noxious sensation and, maybe later in evolution, pain? However, it would be counterproductive if this sensory experience could "cripple" an organism into inactivity. The following scenario might be envisaged: Under serious situations commanding attention, the sensation of pain must subside momentarily (i.e., analgesia) to allow for an appropriate response to the stimulus. While the organism is orienting itself to the stimulus, the simultaneous release of enkelytin, analgesic and immunocyte-stimulating opioid peptides combats the pathogenic challenge of a bacterial presence [8]. Once this is over, the pain-provoking process can emerge once more, possibly even at a stronger level, resulting in behaviors designed to alleviate the condition.

Indeed, if pain evolved to fit this function, it evolved in association with immune processes. Furthermore, these opioid mediated activities were probably enhanced during evolution as the central nervous system (CNS) became closed off from the circulatory system by the blood-brain barrier, ultimately isolating the ganglionic neural activities that also required immune surveillance. However, regardless of the barrier, various immune cells, i.e., those responsive to opioid peptides, were always allowed access to the isolated tissues, some taking up residence in the CNS as microglia. The reason for the evolving relationship between opioid neural and immune processes now appears quite simple, that is, analgesic priority-setting activities associated with an anti-infectious/anti-inflammatory process. This combination would provide a high degree of survival benefit to any organism, since it would ensure appropriate behavior to meet not only these noncognitive challenges, but also cognitive ones [8].

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G    65    CKDLLQVSKQELPQEGASSLRESGKQDESHLLSKKYGGFMKRYGGFMKKVDELYPVEPEE  124

M    24    CK-IFQYRLQKCPSLKASSLRESGKQDESHLLSKKYGGFMKRYGGFMKKVG-----EPE-   76


G   125    EANGGEILTKRYGGFMKKDAEDGDALANSSDLLKELLGTGDDRDRENHHQEGGDSDEGVS  184

M    77    EILTKR-----YGGFMKKD-EAAQAAANSSDLLKELLGTGDDRDRENHHQEGGDSDEGVS  130


G   185    KRYGGFMRGLKRSPQVEDEAKELQKRYGGFMRRVGRPEWWMDYQKRYGGFLKRFAEFLPS  244

M   131    KRYGGFMRGLKRSP---KSRSSEQ---------V--------VQKRYGGFLKRFAEFLPS  170


G   245    DEEGESYSKEVPEMEKRYGGFMRF

M   171    EEEGESYSKEVPEMEKRYGGFMRF
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Figure 1. Comparison of invertebrate and mammalian proenkephalin. Common amino acids are shown in red for comparison with the mammalian material, opioid peptides in blue and the enkelytin (green) sequence, including [Met]enkephalin-Arg-Phe (blue) is underlined, G – Guinea pig, M – *Mytilus edulis*, dashed lines represent spaces in the proenkephalin molecules as determined by a best fit model.
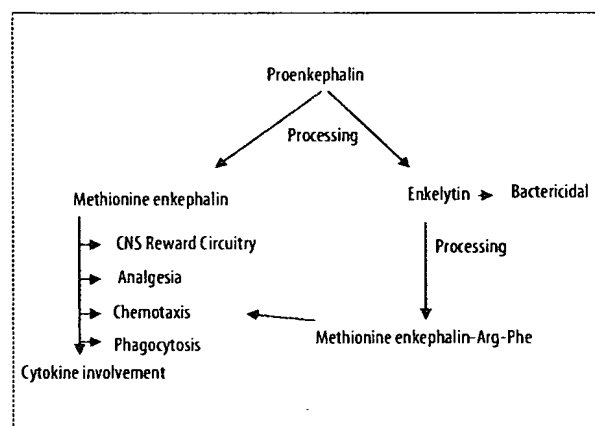
Figure 2. Complementary immune actions of [Met]enkephalin and enkelytin. Bacterial products (e.g., lipopolysaccharide) or tissue trauma (e.g., a cut) can induce the simultaneous release of [Met]enkephalin and enkelytin from immune cells. [Met]enkephalin induces immunocyte chemotaxis and the release of other signaling molecules (i.e., cytokines), whereas enkelytin exerts an antibacterial action. Within minutes enkelytin too is processed to yield [Met]enkephalin-Arg-Phe that further augments the immunocyte response.

Furthermore, given the presence of the same signaling molecules in central nervous system reward circuits, i.e., opioid and opiate molecules [11,12], one can also surmise that here too, processing of proenkephalin leads to an enkelytin presence. Thus, feeling good, indicating opioid peptide possessing, may also create a healthy internal environment and may form the basis of a novel protective survival strategy. Given the belief and trust components of feeling good, involving these signal molecules, this may be the basis of promoting health as can be demonstrated in placebo experiments as well as complementary and alternate medical therapies [11,13–15].

ENDOGENOUS OPIATE ALKALOIDS: MORPHINE

In the past we have noted the many reports documenting the presence of morphine and its metabolites and precursors in animals, including invertebrates, strongly suggesting that this chemical messenger can be made by animals [16–18]. Recently, two reports have emerged noting that the addition of opiate alkaloid precursors results in enhanced morphine levels in cancer cell lines and incubating normal healthy ganglia, demonstrating the synthesis of morphine [19–21]. These studies are complemented by the demonstration of a mu opiate receptor subtype, mu3, that is opiate alkaloid selective and opioid peptides insensitive present on immune, vascular and neural tissues [22].

Functional roles of endogenous morphine

In this discussion of the possible activities of endogenous opiates we are guided by information collected in numerous studies on the pharmacological responses to the administration of exogenous morphine and related drugs. One feature that appears to be characteristic of exogenous opiate compounds, exemplified by their known antinociceptic effects, is that they lower thresholds under a variety of physiological and pathological conditions. It is, therefore, reasonable to speculate that endogenous opiates may act in a similar capacity, wherever a situation calls for it.

The presence of opiate alkaloids in the circulation and of special opiate receptors on immunocytes, demonstrated in vertebrates as well as invertebrates, enables these compounds to participate directly in auto- and immunoregulatory activities [6,16]. Indeed, some reports have noted excitatory effects of morphine, however, in part, we have demonstrated that this may occur from a rebound from inhibition – its just that the observation time points in those studies did not pick up the initial inhibitory effects [23–27]. These direct activities may be judged to be largely of an inhibitory nature. In addition, circulating opiates may contribute to the total sum of directives mediated by signal molecules
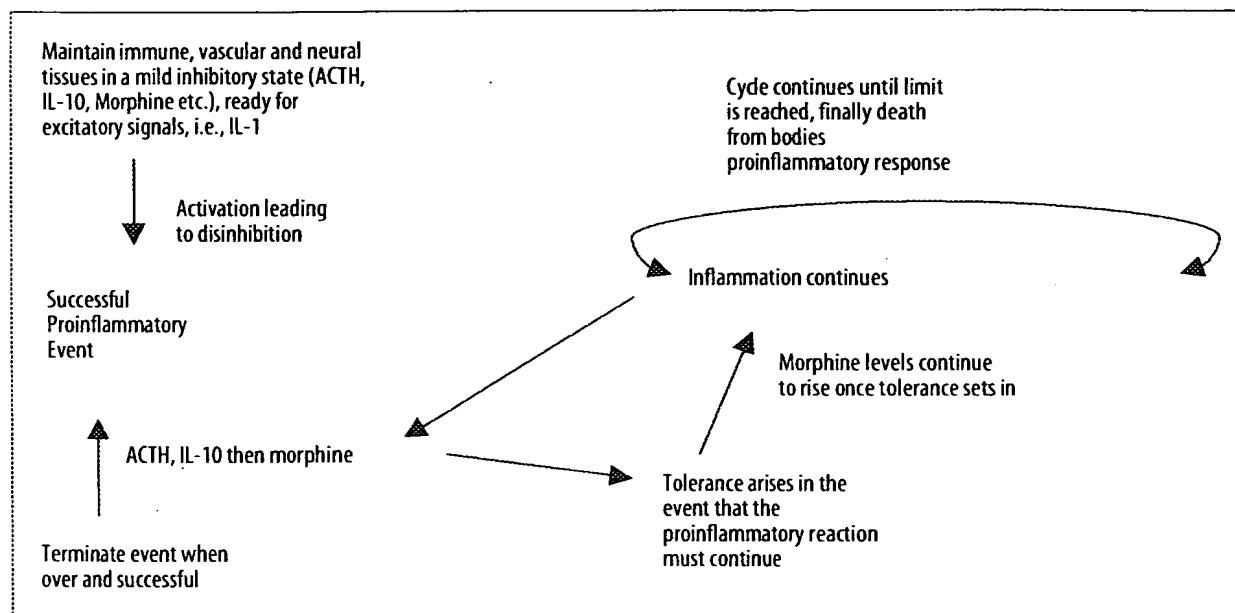
Figure 3. Morphine's natural function to limit excitation. For example, in an immune event, "tonal/basal" morphine levels initially remain low, mildly inhibiting the cellular components. Once a sufficient stimulus is induced/present, the system becomes disinhibited, leading to activation. After this process continues, the body's processes (i.e., brain and adrenal gland) attempt to restore normal function via down regulating signal molecule production, i.e., morphine, which exhibits a latency period before its increased concentration can be detected. However, if excitation continues to rise, the augmented morphine levels go unfelt, i.e., tolerance, allowing the process to continue. We surmise the body repeats this cycle, when more and more activity is called for until the down regulating capabilities are lost, suggesting the individual may succumb to the bodies widespread activation, which when left unchecked becomes toxic, i.e., sepsis.

reaching the central nervous system from various sources, including the immune system.

Another, i.e., indirect, pathway for the suppression of immune processes by morphine can be postulated, namely that via the hypothalamic-hypophysial-adrenal axis (Figure 3). In this case, the final outcome, immunosuppression, must be visualized to be initiated by a stimulatory signal from morphine, furnished by the brain, to CRH-producing neurons of the hypothalamus, the first way station in this axis. This concept is supported by the immunocytochemical demonstration of a morphine-like compound in the rat hypothalamus as well as morphine's action in releasing CRF, which would then enhance plasma ACTH levels followed by that of glucocorticoid [28–30].

The direct and indirect down regulating activities attributed to endogenous morphine should be considered in the context with those of other chemical mediators known to act in the same capacity. One of these inhibitory molecules is the cytokine interleukin-10 (IL-10), which is released by macrophages to counteract excessive immunostimulation caused by other cytokines which, under certain conditions of activation, are produced and released by the same cells (see [31,32]). Another inhibitory signal molecule produced by immunocytes is ACTH [33], which, like IL-10, can be considered to participate in autoimmunoregulatory activities and can be stimulated by morphine [30].

The question arises in which manner and under which circumstances the immunosuppressive activity of endogenous morphine is called into action that can be interpreted as

positive for the organism. It is reasonable to speculate that the need for an additional control system may arise under conditions making unusual demands.

There seems to be general agreement on the fact that serious or life threatening challenges create a state of alertness, brought about by the instant release of stimulatory messenger molecules (opioid peptides and others), during which all available energies are directed toward meeting the emergency [1,34]. What should be considered to be equally important is that these stimulatory signals need to be stopped as soon as they are no longer required, so as to prepare the organism for a subsequent challenge. Endogenous morphine would seem to be an appropriate candidate to meet this demand.

For example, during major surgical interventions, the immunosuppressive effect of ACTH and IL-10 produced by immunocytes may not suffice to lower the hyperstimulation of granulocytes and macrophages attributable to their release of IL-1 and TNF due to this trauma. It seems reasonable to suggest that, under these circumstances, morphine may be called upon to down regulate the process so as to restore the normal level of activity [34]. The validity of this proposal is supported by tests carried out with blood samples taken from patients during cardiopulmonary bypass operations. In preparations exposed to morphine, signs of cellular activity were less pronounced than in untreated samples, and plasma morphine levels in surgical patients were high, following a 24 hour latency period [35,36].

The results of another experiment, indicative of the down regulating capacity of endogenous morphine under conditions

of stress, deserve attention, since it was first reported in an invertebrate. The design was to follow the sequence of activities generated by subjecting the mollusc Mytilus edulis to stressful interventions (electrical shock, prevention of valve movements [37]). The immediate response was activation of the animal's defense system, as judged by conformational changes in its immunocytes, and interpreted to be brought about by the release of endogenous opioid peptides and additional molecules. Twenty-four hours later, when the state of alertness had subsided, the return of the immunoactive hemocytes to a more "inactive" conformation was found to concur with a temporary but significant rise in the opiate content of nervous tissue and hemolymph [35,38]. The conformation of the immunocytes observed at this point in time resembled that of unstressed animals exposed to exogenous morphine.

The insights gained from the study of the various traumatic situations cited suggest that in the heirarchy of available down regulating mechanisms, morphine operates as a strong backup system. The observation that this secondary system goes into effect after a latency period during which endogenous opiate levels rise, is in line with the fact that the μ_3 opiate receptor has an affinity constant in the range of 10^{-8} M [22,39].

Evidently, the availability of a network of effective immunostimulatory agents has great survival value for vertebrates and invertebrates alike. It is, therefore, understandable that the development of its elements, including those operating in immunoregulation, can be traced far back on the evolutionary scale. The need for the operation of more than one immunosuppressive mechanism is as obvious as that for the availability of effective immunostimulatory agents. It is our belief that it is one of morphine's important tasks to meet this vital demand.

In addition to those discussed here, endogenous morphine and related opiates may be presumed to engage in a variety of other activities, for example some operating within the confines of the nervous system [18].

TOLERANCE

With the above discussion in mind we will now consider the phenomenon of tolerance with regard to endogenous opiate alkaloid compounds, morphine, morphine-6-glucuronide, etc. since they too are endogenous to various animal tissues, including invertebrates [40–43]. With continued exposure to the same dose of an opiate, various physiological systems exhibit a decrease in their response. This phenomenon is referred to as tolerance. As with the study of opiates, our historic interest in this phenomenon is focused around anti-nociception. However, given that morphine is a naturally occurring signal substance, we must ask another question. Since tolerance occurs, what would its "normal" function be? We have examined the need for 'turn-on' molecules as well as the need for 'turn-off' molecules in various systems. Now we must consider what turns off or down regulates this 'off' system, i.e., morphine.

We believe the answer, in part, to this question is in the phenomenon of tolerance. Once the down regulatory process has been initiated and the level of these molecules (i.e., morphine) rise to competitively overcome the influences of the

initial stimulatory molecules, the inhibitory molecule's level, i.e., morphine (as also noted by the K_d for morphine on the mu3 receptor, approx. 15–50 nM), would be hard to overcome, as would be its continued presence due to its relatively long half-life as well as its conversion to morphine-6-glucuronide, extending its actions longer in time [44]. Thus, stimulatory signal molecules could not activate the system during this down regulation unless their concentrations rose well above those levels in the initial event. Indeed, at this moment, the activities generated by an additional phase of excitatory molecule release would upset the now instituted down regulation, given its competitive and reversible signal molecule nature. This is especially true of morphine [44]. However, the only mechanism that can effectively diminish the inhibitory actions over a relatively short period would be one in which the very same effector cell system progressively becomes desensitized to the presence of morphine. In this way, the down regulating influence would be terminated regardless of the concentration of morphine present during a single event. Thus, the effector cells become tolerant. Interestingly, tolerance would only set in once down regulation had been achieved, because desensitization relies on higher or extended morphine concentrations – a dose that would have already exerted its down regulating properties before. Thus, the 'brake' would be administered on a 'need' basis along with the dynamic capability of progressive adjustments in this process if required. Moreover, tolerance, once achieved, also would allow for further stimulation of the system if it was required, since morphine's presence would not be "sensed".

In summary, tolerance represents a dynamic mechanism that can be used to augment various regulatory processes whether they are involved in excitation or inhibition. Simply stated, tolerance is a process that allows for the termination of morphine's action while it is still present in the environment. It is still present in the environment because it is a general, yet specific, mechanism operating only at concentrations above basal levels, concentrations that would terminate excitatory processes. However, tolerance ensures that immuno-inhibition, for example, does not last to the point whereby the organism would be compromised due to a lack of a functioning system, i.e., immune, over an extended period of time. Thus, desensitization sets in and allows the various processes to be stimulated and operational once more. Indeed, there is a rebound into excitation from inhibition that also occurs with morphine [24–26,45]. Clearly, the timely 'rebound' of the immune and nervous processes involved with opiate actions provides for a successful mechanism to ensure survival as has been demonstrated (see [23]). Since tolerance has been demonstrated in invertebrates and vertebrates, the use of this strategy becomes even more evident.

Dependence/addiction

In drug dependence, one can see tolerance develop with a decreased response to the actions of a drug. Thus, in order to achieve the same effect one has to take a larger dose. The phenomenon of dependence occurs upon the withdrawal of the drug, which produces behavioral signs opposite of those desired. Furthermore, associated psychological dependence involves compulsive drug-seeking behavior (i.e., craving) as well as the diminishing of normal motivators, i.e., health issues [12]. In all animals there are normal behaviors which can be said to be based on a compulsive 'foundation'. It

Table 1. Phenomena that may trigger or represent the basis of addiction. Immune cells in malignant histyocytosis where found to be devoid of opiate receptors and did not respond to morphine, that is, becoming down regulated [50]. Exercise has the ability to increase endogenous plasma levels of morphine [51].

Addiction
1. Morphine Insufficiency Syndrome: Individuals may not be making enough morphine and/or opiate receptors may be faulty or not present, and when they first experience it they feel normal. This may induce normal behaviors that augment endogenous morphine processes/levels, i.e., exercise. In time, tolerance may set in, causing an increase in this behavior, which may become compulsive. The other possibility is that after experiencing external morphine they directly seek it, restoring normality. However, tolerance and dependency will eventually set in because this external source cannot be fine tuned.
2. External sampling of morphine, i.e., for pleasure or analgesia, which bypasses the normal and appropriate reward and/or pain circuitry, triggering the cycle of tolerance and finally dependency upon continuous use.

would be interesting to speculate that "addiction" emerges from tolerance if the presence and level of endogenous opiates do not or cannot return to their previous or pre-stimulation low levels. In this scenario, once tolerance occurs, the opiate molecules, i.e., morphine, remain relatively elevated. In this event, in order to further lower the threshold of activation, e.g., immunosuppression, one would require even greater increases in morphine levels due to tolerance. It would be to an organism's benefit to have such an immune mechanism, since this would allow for a more dynamic response to antigenic challenge, for example. Thus, an animal would have several levels of immune responsiveness to call upon. Indeed, we surmise, the lack or dysfunction of such a system may lead to various pathologies, i.e., hyperactive cell disorders, autoimmune disorders, etc.

However, given the above, the dynamics of passing through many levels of tolerance may adversely affect the organism. Clearly, operating at different morphine levels may force a "system/process" to continually re-establish its threshold for excitability due to tolerance setting in. We surmise that at critical developmental periods, this process may also leave a permanent change in neurological and immune systems [12]. This endogenous trauma may manifest itself, as noted above, in the display of opposite "behaviors" i.e., excitability of nerve or immune cells. Indeed, one may associate immune stimulation with morphine actions due to the fact the process has become tolerant and thus supersensitive when morphine availability ceases [23]. In a concurrent neuronal morphine receptor supersensitive state, if the stimuli/state became cognitive, one would actively seek out opiates. In this regard, addictive or compulsive behavior may be viewed as a process indicative of morphine insufficiency. A subpopulation of individuals seeking for continuously higher levels of opiates (i.e., those addicted) may be operating from a morphine insufficiency status. Additionally, these individuals may also exhibit lower concentrations or densities of mu receptors, since tolerance and other factors possibly induce a downregulation of related opioid receptors, leading to the need for higher opiate concentrations, i.e., morphine, for a normal physiology to operate. Therefore, addictive behavior may be viewed as a phenomenon that may reflect opiate insufficiency in an organism with attendant alterations in neuro- and immunoregulation. Indeed, what may initiate the cascade of tolerance in a potential addict, an individual who may have an endogenous morphine biosynthesis and/or excessive degradation problem, is the very first experience with such a substance (Table 1). For in this experience, a subpopulation of individuals may

for the first time 'feel' normal. This might reflect the 'neurological susceptibility' to drug addiction referred to by Dole and Nyswander in their 1967 metabolic theory of addiction [46]. Normal is defined as being in the sense a diabetic feels being given insulin [47]. However, in the morphine insufficiency scenario, as morphine is administered, tolerance develops because it is the normal way endogenous morphine's presence is down regulated. Thus, persons enter into this cycle of dependence as they continually seek to regain and maintain the 'normal' feeling – often overshooting into a state of intoxication. This hypothesis offers the explanation for the phenomenon that some individuals treated for drug addiction are not 'cured'. In this specific case, the reason may simply be that the endogenous opiate insufficiency has not been addressed, and by providing the substance one compromises the existing regulatory processes, i.e., tolerance.

CONCLUSIONS

Taken together, endogenous morphine appears to exert positive effects on health by limiting the degree of excitation. Supporting this view is the fact that the mu3 opiate receptor subtype is coupled to constitutive nitric oxide release, which also appears to have this down regulating action in neural, immune, vascular and gastrointestinal tissues [16,48,49]. Thus, morphine down regulates tissue processes in addiction, an action/function that it may normally perform when the situation calls for this appropriate function and by so doing in this natural setting, sustain life.

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Review

Opioids, opioid receptors, and the immune response

Lois McCarthy [a], Michele Wetzel [a], Judith K. Sliker [a], Toby K. Eisenstein [a],
Thomas J. Rogers [a,b,*]

[a] Department of Microbiology and Immunology, and Center for Substance Abuse Research, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140, USA
[b] Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140, USA

Abstract

It is now clear that opioid receptors participate in the function of the cells of the immune system, and evidence suggests that opioids modulate both innate and acquired immune responses. We review literature here which establishes that μ-, κ-, and δ-opioid compounds alter resistance to a variety of infectious agents, including the Human Immunodeficiency Virus (HIV). The nature of the immunomodulatory activity of the opioids has been the subject of a great deal of research over the last ten years. There is increasing evidence that effects of opioids on the immune response are mediated at several levels. Modulation of the inflammatory response appears to be a target of these compounds, including effects on phagocytic activity, as well as the response of cells to various chemoattractant molecules. Moreover, findings from several laboratories have demonstrated the impact of opioid treatment on antibody responses, and the molecular basis for this effect is likely due, at least in part, to the modulation of both cytokine and cytokine receptor expression. Future research should provide a clearer understanding of the cellular and molecular targets of opioid action within the immune system. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Opiate abuse; Immune function; Immunobiology; Molecular biology; HIV review

1. Introduction

There is a rapidly growing literature which has described a broad range of effects of opioid compounds on the function of cells of the immune system. It is clear that the immunomodulatory activity of opioids is likely to be due to a complex interplay of direct and indirect influences. In the whole animal, opioid modulation of the immune response is mediated, in part, directly through the interaction with opioid receptors expressed by one or more populations of immune cells. In addition, the influence of the opioids on the immune response in vivo is likely to be also the result of the participation of both the central nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. The re-

sults of studies carried out in the whole animal must be evaluated with the understanding that the brain, adrenal, and multiple sub-populations of immune cells may each play a role in the final outcome. This review will concentrate largely on the direct interaction of opioids with the cells of the immune system.

2. Pharmacological characterization of the leukocyte receptors

Pharmacological evidence has been reported which demonstrates the presence of μ-, κ-, and δ-opioid receptors, as well as non-classical opioid-like receptors, on cells of the immune system. Mehrishi and Mills (1983) reported naloxone binding to human lymphocytes and platelets, and this binding was found to be both specific and sensitive to competition with morphine. Studies

* Corresponding author. Tel.: +1-215-7073215; fax: +1-215-7073218.
E-mail address: rogerst@astro.ocis.temple.edu (T.J. Rogers).

carried out with human polymorphonuclear leukocytes have shown the presence of a stereospecific binding site for naloxone (Falke et al., 1985). More recently, Ovadia et al. (1989) have demonstrated two specific and displaceable naloxone-binding sites on rat T-lymphocyte membranes. These naloxone-binding sites differed on the basis of affinity (Kd = 10 nM and > 20 nM) and appeared to be expressed only after activation of the T-cells.

Human granulocytes and monocytes appear to express a μ-like opioid receptor based on the specific and saturable binding of dihydromorphine (Lopker et al., 1980). These studies suggest a binding affinity of approximately 10 nM, with 3000–4000 binding sites per cell. Radulescu et al. (1991) have reported specific murine splenocyte binding of the μ-selective agonist 2-(p-ethoxy-benzyl)-1-[N,N-diethylamino]

-ethyl-5-isothiocyanatobenzimidazole (BIT) and analysis suggests the presence of 3000–30 000 sites per cell. The range of binding sites per cell may in part reflect the heterogeneous nature of the cells which populate the spleen.

The macrophage-like murine cell line P388D$_1$ has been shown to possess enantioselective κ-like opioid binding sites (Carr et al., 1991) with a binding affinity in the nanomolar range. The binding of the κ-selective agonist U69 593 was shown to be partially displaced by the κ-selective agonist dynorphin (1-13) and the opioid antagonist naltrexone (Carr et al., 1989). However, the U69 593 binding was only weakly displaced by the δ-selective agonist met-enkaphalinamide or the μ-selective agonist [D-ala^2, N-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO). A κ-like specific binding site has also been detected on the murine T-cell line EL-4 using the κ-agonist bremazocine (Fiorica and Spector, 1988). The binding of bremazocine was not stereospecific. However, the binding was readily displaceable by κ-, but not δ-opioid agonists. In addition, the EL-4 κ-binding site exhibited a dissociation constant of approximately 60 nM.

Finally, the presence of δ-like receptors on cells of the immune system has been reported by several investigators. Human granulocytes have been shown to possess δ-selective binding sites based on the specific binding of ethylketocyclazocine, diprenorphine, DADLE, and [D-Ala^2]-deltorphin (Falke et al., 1985; Stefano et al., 1993). The binding of [D-Ala^2]-deltorphin was found to be readily displaceable with the δ-selective agonists met-enkephalin and [D-Ala^2,Met^5]enkephalinamide, or the opioid antagonist naltrexone, but not with the μ- or κ-selective agonists DAMGO or dynorphin(1-17), respectively (Stefano et al., 1993). A δ-opioid-like receptor has also been described on the human T-cell line Jurkat, as well as the murine macrophage cell line P388D$_1$ (Ausiello and Roda, 1984; Carr et al., 1989). The binding of leu-

enkephalin to the Jurkat cell line was saturable and specific; but no detectable binding-inhibition was observed with the antagonist naloxone (Ausiello and Roda, 1984). The nature of the unusual binding properties for this cell line remains uncertain.

A non-classical opioid-like binding receptor present on cells of the immune system has been identified by several laboratories. This binding site appears to possess some specificity for the endogenous opioid β-endorphin, but is naloxone insensitive (Hazum et al., 1979). In addition, the binding to the EL-4 cell line is not inhibited by either leu- or met-enkephalin, or by the amino-terminal fragments β-endorphin (1-16) or β-endorphin(1-27) (Schweigerer et al., 1985). These results and others have led to the conclusion that the binding to this non-classical binding site is dependent on the carboxyl-terminal segment (Hazum et al., 1979; Schweigerer et al., 1985).

The β-endorphin receptor has also been identified on the transformed human lymphocyte line RPMI 6237 (Hazum et al., 1979), murine splenocytes (Shahabi et al., 1990) and bone marrow macrophages (Gelfand et al., 1995), the murine B-cell line A20 (Shaker et al., 1994), and the human monocyte cell line U937 (Shahabi et al., 1990). Binding studies suggest that the affinity of the receptor for β-endorphin varies between 0.3–0.4 nM for the RPMI 6237 transformed human lymphocyte line and murine splenocytes, and 65 nM for the EL-4 T-cell line. The A20 cell line exhibits both a very high affinity receptor (Kd = 87 pM) as well as a lower affinity binding site (Kd = 22 nM) (Shaker et al., 1994). Treatment of the A20 cells with the lectin concanavalin A results in a loss of the high affinity receptor along with an increase in the expression of the low affinity receptor. This result suggests that the state of activation or differentiation of the cell may have an impact on the expression of a high affinity form of the β-endorphin receptor.

3. Molecular characterization of the leukocyte opioid receptors

Each of the major opioid receptor classes have now been cloned from neuronal cells and fully sequenced. These proteins are seven transmembrane receptors and share homology with the somatostatin receptor (Reisine and Bell, 1993). Analysis of the δ-opioid (Evans et al., 1992; Kieffer et al., 1992), κ-opioid (Li et al., 1993; Minami et al., 1993; Nishi et al., 1993; Yasuda et al., 1993; Zhu et al., 1995) and μ-opioid (Chen et al., 1993) sequences show a high degree of amino acid homology among the opioid receptors. Northern blot analysis has shown that the δ-opioid receptor mRNA varies in size between 1.4 and 9 Kb (Evans et al., 1992). In a similar fashion the κ-opioid receptor mRNA isolated from

brain tissue is quite large (5.2–6.0 Kb) (Yasuda et al., 1993; Zhu et al., 1995), as is the μ-opioid mRNA (> 10 Kb) (Raynor et al., 1995). The large size of these transcripts is due to a long 3' untranslated region in each case.

The genomic organization of neuronal opioid receptors is now being clarified. Both the μ- and μ-opioid receptors appear to possess three exons with multiple transcription initiation sites identified (Min et al., 1994; Augustin et al. 1995). The κ-opioid receptor was first reported to be composed of three exons (Nishi et al., 1994), however, more recent reports have clearly established the existence of an additional exon 5' of the exon containing the translation start site (Belkowski et al., 1995a; Liu et al., 1995; Yakovlev et al., 1995).

While involved in the cDNA cloning of the μ-, κ-, and δ-opioid receptors, several investigators have cloned and sequenced an opioid-like receptor which fails to bind to the opioid agonists commonly employed to characterize the classical opioid receptors (Fukuda et al., 1994; Mollereau et al., 1994). This receptor possesses homology with the other opioid receptors and was originally termed the orphan opioid receptor. More recently the natural ligand for this receptor was identified as a heptadecapeptide which resembles dynorphin A and has been termed nociceptin (Meunier et al., 1995; Reinscheid et al., 1995). The genomic structure of the nociceptin receptor is organized into three exons, and the translated sequence is approximately the same size as the cloned opioid receptors (Nishi et al., 1994).

Efforts have recently been made to examine the molecular structure of the opioid receptors expressed by cells of the immune system. The evidence reviewed above suggested that the opioid receptors expressed in these cells may possess unique features. A partial sequence for the δ-opioid receptor was recently obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) from monkey lymphocytes (Chuang et al., 1994). Examination of the partial sequence of this receptor revealed essentially complete identity with the brain δ-opioid receptor. A partial sequence for the μ-opioid receptor has also been reported for rat peritoneal macrophages (Sedqi et al., 1995). Here again, the sequence is essentially identical to the brain μ-opioid receptor. And finally, a partial κ-opioid receptor sequence has been obtained from human and monkey lymphocytes (Chuang et al., 1995). The results from each of these studies strongly support the presence of classical opioid receptors expressed by cells of the immune system.

A report by Belkowski et al. (1995a) first described a full-length sequence of an opioid receptor expressed by cells of the immune system. These investigators employed the immature T-cell lymphoma cell line R1.1 as a source of mRNA for RT-PCR analysis and identified the presence of the κ-opioid receptor sequence. This cell line has been shown previously to possess typical κ-opioid receptors based on pharmacological analyses (Bidlack et al., 1992; Lawrence and Bidlack, 1992, 1993).

RT-PCR was performed with RNA from the R1.1 cell line using several oligonucleotide pairs permitting analysis of the the full-length sequence, as well as the sequence of individual regions of the open reading frame. Analysis of overlapping RT-PCR fragments and the full-length product revealed a surprising degree of heterogeneity at the 5' end of the coding region. Nucleotide sequences of multiple clones of the PCR products from the R1.1 thymoma cells were determined, and assembly of the individual overlapping PCR products and the sequence of multiple full-length clones confirmed the heterogeneity in the region of the κ-opioid receptor near the start codon. Clones were observed with a sequence which is essentially identical (99.8% nucleotide homology; 100% amino acid homology) to that of the reported murine brain κ-opioid receptor (Yasuda et al., 1993). Clones were also identified with a 30-bp insertion 15 bp upstream of the initiation codon. This 30-bp insertion is present in the cDNA of rat κ-opioid receptors (Li et al., 1993).

The presence of the 30-bp insertion in the 5'-noncoding region was somewhat surprising given the initial reports of the genomic structure of the mouse μ-, κ-, and δ-opioid receptors (Min et al., 1994; Nishi et al., 1994; Augustin et al., 1995). Within the coding regions, there were two introns reported for the δ and κ receptors and three introns in the μ receptor. The 30-bp insertion suggested alternative intron-exon splice variation which could only occur if an additional exon 5' of the exon containing the initiation codon is present in the genomic sequence. As stated above, more recent reports have clearly identified the sequence of this fourth exon in the κ-opioid receptor (Nishi et al., 1994). It is interesting that this region of the first intron is normally included as a part of the second exon in the prominent transcript identified in rat brain tissue (Yakovlev et al., 1995).

These results suggest that multiple κ-opioid receptor mRNA species are present in the R1.1 cell line, and either the κ-receptor or the cell population may be unique in this regard. The impact of these inserted sequences in the 5'-noncoding region on expression of the κ-opioid receptor remains uncertain at this time. Additional analyses of other cell lines as well as primary cells to determine whether any differences are seen in the sequence of the opioid receptors of these cells may provide more information on the interaction seen between the brain and immune system.

Further analysis of the R1.1 cell line has resulted in the identification of an additional intron-exon splice variant (Alicea et al., 1998). This splice variant fails to utilize any part of the second exon for the mature transcript. Translation of this variant murine transcript

would be expected to result in the lack of the entire amino-terminal region, the first transmembrane domain and part of the first intracellular loop. There is an in-frame potential translation initiation site near the 5′ end of the third exon, and therefore this translation product would retain part of the first intracellular loop and the remainder of the receptor. This alternate splice variant was detected in the R1.1 cell line as well as primary macrophages and brain tissue. The level of expression of this truncated transcript relative to the full length transcript appears to vary among tissues. Examination of numerous RT-PCR reactions suggests that the truncated transcript is almost as abundant as the full-length transcript in the R1.1 and DPK immature T-cell lines, and is the only form detected in primary murine macrophages. On the other hand, the truncated transcript is present at roughly 10-fold lower levels in the brain. These splice variants may provide a source of protein heterogeneity and could explain some of the unusual binding properties by the immune cells.

A variant κ-opioid receptor transcript in rat brain tissue has also been described (Yakovlev et al., 1995). This variant transcript is missing the first exon and begins within the first intron and contains two potential translation initiation codons which are in-frame with the typical initiation codon for the κ-opioid receptor. Translation from these additional initiation codons may result in the formation of alternative receptor proteins which contain longer novel amino acid sequences at the amino termini.

The formation of splice variants has also been observed for both the μ-opioid and orphanin FQ/nociceptin (formerly the orphan opioid) receptors (Bare et al., 1994; Wang et al., 1994; Wick et al., 1994; Halford et al., 1995; Zimprich et al., 1995; Mayer et al., 1996). Bare et al. (1994) identified a human brain μ-opioid receptor splice variant in which the 3′ terminal intron was not removed, and based on the location of an in-frame termination codon, this variant would be expected to be eight amino acids shorter and contain four distinct carboxyl-terminal amino acid residues (VRSL) when compared with the full length μ-opioid receptor. Zimprich et al. (1995) have identified a rat variant μ-opioid receptor which is seven amino acids shorter and contains five distinct carboxyl-terminal amino acids (KIDLF) compared to the prominent μ-opioid receptor. These forms of the rat μ-opioid receptor appear to have similar binding properties and are equally effective in inhibiting forskolin-stimulated cAMP production. It is very interesting that this splice variant of the rat μ receptor is much more resistant to agonist-induced desensitization (Zimprich et al., 1995). It is possible that this splice variant of the rat μ-opioid receptor is derived from an alternative fourth exon (Mayer et al., 1996).

A splice variant of the 1.5 kb lymphocyte orphanin FQ/nociceptin receptor (missing 15 bases) has also been identified in both mouse lymphocytes and the brain (Halford et al., 1995). The splice variant occurs in 45–50% of the receptor species in lymphocytes, whereas the variant occurs in only 25–30% of the receptor species in brain. This splice variant is missing amino acids that are part of the first intracellular loop and may constitute a region important for signal transduction. Another splice variant containing an additional 84 base pairs between the third and fourth exons of the orphanin FQ/nociceptin receptor has also been identified in rat brain (Wang et al., 1994). In this case the alteration would be expected to yield an additional 28 amino acids in the third extracellular loop. The impact of these changes in the receptor sequence on the binding or signaling properties remains to be determined.

4. Effect of opioids on immune cell function

4.1. Phagocytic cell function

The capacity of both peritoneal macrophages and neutrophils to phagocytose the pathogenic yeast *Candida albicans* is inhibited following in vivo administration of morphine (Tubaro et al., 1983, Rojavin et al., 1993, Pacifici et al., 1993). Studies reported by Rojavin et al. (1993) with non-elicited macrophages suggest that the inhibitory activity of morphine is blocked by the administration of the opioid antagonist naltrexone. This is in contrast to the results obtained by Tubaro et al. (1983) with cells elicited with thioglycolate; and the disparity in these observations is likely to be due either to the timing of the antagonist administration or the elicitation of the phagocytic cell population.

The phagocytosis of sheep erythrocytes by elicited macrophages is inhibited in vitro by administration of either morphine or the endogenous opioids leu- and met-enkephalin (Casellas et al. 1991). Studies carried out by Szabo et al. (1993) show that non-elicited macrophages treated with morphine also exhibit a reduced capacity to ingest *C. albicans*. Using selective antagonists it was determined that the effect of morphine in vitro was due primarily to the ability of this opioid to act through the μ-opioid receptor. Additional studies showed that the phagocytic activity of non-elicited macrophages is inhibited by in vitro administration with μ-, κ-, or δ-opioid agonists (Szabo et al., 1993). The inhibitory activity of each agonist is blocked by an antagonist with the appropriate opioid receptor selectivity.

In contrast to the inhibition of phagocytic activity following opioid administration, morphine and the endogenous opioids β-endorphin, met-enkephalin, and dynorphin induce chemotaxis of human monocytes and neutrophils (Van Epps and Saland, 1984; Makman et al., 1995; Ruff et al., 1985; Grimm et al., 1998). On the

other hand, pre-treatment with opioids leads to an inhibition in the chemotaxis of neutrophils and monocytes to both complement-derived chemotactic factors (Liu et al., 1992; Perez-Castrillon et al., 1992) or to the chemokines MIP-1α, RANTES, MCP-1 or IL-8 (Grimm et al., 1998). In these studies the administration of morphine, heroin, met-enkephalin or the more selective μ-agonist DAMGO or the selective δ-agonist [D-Pen², D-Pen⁵]enkephalin (DPDPE) was found to inhibit the subsequent chemotaxis of human peripheral blood neutrophils or monocytes. The results of Grimm et al. (1998) suggest that the activation of the μ- and δ-opioid receptors leads to the desensitization of the chemokine receptors CCR1, CCR2, CXCR1 and CXCR2. This opioid-induced desensitization appears to be due to the phosphorylation of the chemokine receptor. These studies contrast with the results of Simpkins et al. (1984) who found that pre-treatment with β-endorphin enhanced the chemotaxis directed to N-formyl-methionyl-leucyl-phenylalanine (FMLP). The disparity in these results is not immediately obvious but may be due to the capacity of β-endorphin to interact with a receptor distinct from the classical opioid receptors (Hazum et al., 1979).

In related studies, morphine was found to potently inhibit the directed migration of microglial cells toward C5a in a dose-dependent manner (Chao et al., 1997). In addition, the mu selective agonist DAMGO also exhibited a dose-dependent suppression of microglial cell chemotaxis. These findings suggest that activation of the constitutively expressed mu opioid receptor (MOR) inhibits microglial cell chemotaxis and support the notion of an anti-inflammatory role of MOR within the brain.

A study reported by Sharp et al. (1985) has demonstrated that the opioids β-endorphin and dynorphin stimulate superoxide production by both neutrophils and peritoneal macrophages. On the other hand, pre-treatment with leu- or met-enkephalin reduced the ability of neutrophils to generate superoxide in response to FMLP (Simpkins et al., 1986). These results were extended by Peterson et al. (1987a, 1989) who showed that concanavalin A- or phorbol myristate-induced superoxide production by human peripheral blood mononuclear cells was inhibited by treatment with morphine. It is possible that the inhibition of superoxide production by morphine may be due to the production of soluble factors (possibly transforming growth factor β) by lymphoid cells present in the peripheral blood preparation (Chao et al., 1992). The impact of opioids on cytokines will be discussed below.

4.2. Lymphoid cell function

The administration of morphine in vivo results in reduced capacity to generate antibody in response to

sheep erythrocytes (Lefkowitz and Chiang, 1975; Bussiere et al., 1992a,b; Bhargava et al., 1994) and to tetanus toxoid (Eisenstein et al., 1990). In most strains of mice, morphine given in vivo induces profound suppression of antibody formation that is blocked by naltrexone, indicating the involvement of a classical opioid receptor (Bussiere et al., 1992a,b). Immune cells treated in vitro with morphine or trans-3,4-dichloro-N-methyl-N-[7-(1-pyrroliidinyl) cyclohexyl] benzene-acetamide methanesulfonate (U50,488), the κ-opioid agonist, also show dose-dependent inhibition of the capacity to mount a secondary antibody response to sheep erythrocytes, which is blockable by pre-treatment with the appropriate opioid receptor-specific antagonist (Taub et al., 1991). The observation that spleen cells of CXBK mice, which are deficient in the μ-opioid receptor, are suppressed by morphine, but not U50,488H, argues for a classical opioid mediated mechanism of immunosuppression. Of interest is the discovery of mouse strain differences in in vitro responses of antibody-forming spleen cells to morphine and U50,488H (Eisenstein et al., 1995).

Attempts to determine the mechanism by which opioids inhibit the antibody response are complicated by the fact that this response is the result of the interaction of several distinct cell populations. Experiments carried out with isolated populations of immune cells have demonstrated that the inhibitory activity of the κ-opioid agonist, U50,488H, is mediated directly through both macrophage (accessory cell) and T-cell populations (Guan et al., 1994). This result is consistent with studies reported by Bussiere et al. (1993) which showed that the inhibition of antibody responses following morphine administration in vivo can be reversed by the addition of non-opioid treated macrophages or the macrophage products IL-1, IL-6, or by interferon-γ.

Studies on the direct effects of opioids on the individual functions of T-cells or B-cells are complicated by the requirement for other cell populations in order to manifest most T- or B-cell responses. Numerous studies have shown effects of opioids on T-cell or B-cell activities, but an alteration in the function of an accessory cell population could explain many of these results (a review of this literature was recently published [Eisenstein and Hilburger, 1998]). At the same time, a recent study by Shahabi and Sharp (1995) showed that the proliferative responses to anti-CD3 of both CD4+ and CD8+ murine splenocytes were directly inhibited by administration of δ-opioid agonists. Due to the purity of the purified T-cell populations used in this latter study, it is likely that the inhibitory activity observed is due to a direct interaction of the δ-opioid with T-cells. The identity of cell populations which express each of the opioid receptor types should provide valuable additional information regarding the cellular targets of the immunomodulatory effects of opioids.

4.3. Cytokine production

Because the regulation of immune and inflammatory responses is dependent on the functions of cytokines, much effort has been placed on understanding the effect of opioids on cytokine activity. Brown and Van Epps (1986) showed that both β-endorphin and met-enkephalin elevated IFN-γ production by concanavalin-A-stimulated human PBMCs. However, the increase in IFN-γ production was not reversible by the opioid antagonist naloxone. Mandler et al. (1986) also found that β-endorphin increased IFN-γ production from PHA-stimulated human large granular lymphocytes, and this effect was partially blockable with naloxone. On the other hand, Peterson et al. (1987b) observed a naloxone reversible suppression in IFN-γ production when human PBMCs were pretreated for 3 h with either β-endorphin or morphine followed by Con-A stimulation.

Bessler et al. (1990) showed that pretreatment with β-endorphin significantly enhanced IL-1-induced IL-2 production in two murine lymphoma cell lines, and that this effect was found to be naloxone reversible. In addition, pretreatment of rat spleen cells with β-endorphin followed by a 24 h stimulation with Con-A increased both IL-2 production and IL-2 receptor α-chain expression (Van Den Bergh et al., 1991). However, the latter effects were not reversed by naloxone, suggesting the participation of a non-classical opioid receptor. In contrast to these results, a dose-dependent inhibition of IL-2 and IL-4 was observed when murine splenocytes were incubated for 24 h with relatively high concentrations of morphine and then stimulated with Con-A (Jessop and Taplits, 1991). Lysle et al. (1993) observed that morphine injected subcutaneously into rats induced a naltrexone-reversible, dose-dependent suppression of splenic lymphocyte IL-2 and IFN-γ production. Chao et al. (1992) demonstrated a naloxone-reversible increase in TGF-β production following morphine treatment of LPS- or PHA-stimulated PBMCs. The inhibition of IL-2 and IFN-γ production following morphine administration may be explained by the well documented immunosuppressive activity of TGF-β.

Studies carried out by Belkowski et al. (1995b) showed that the kappa-opioid receptor agonist U50,488H decreased the LPS-induced levels of IL-1 and TNFα produced by the macrophage-like cell line P388D$_1$. More recently, Alicea et al. (1996) demonstrated that non-elicited pertoneal macrophages stimulated with LPS and treated with U50,488H at concentrations as low as 1 nM exhibited decreased production of IL-1, TNFα and IL-6. On the other hand, LPS-stimulated murine peritoneal macrophages pretreated with a low dose of morphine (50 nM) exhibited a naloxone-reversible increase in TNFα and IL-6. In contrast, those receiving a high dose of morphine (50

µM) exhibited a decrease in IL-6 and TNFα which was not reversible with naloxone. It was determined that low doses of morphine augment LPS-induced NF-κB levels whereas high doses of morphine reduced NF-κB levels suggesting that opioids may be affecting cytokine production at the transcriptional level (Roy et al., 1998).

Shahabi and Sharp (1995) observed that murine splenic CD4$^+$ T-cells treated with the δ-opioid receptor agonist deltorphin and then stimulated with plate-bound anti-CD3 exhibited an increase in IL-2 production when deltorphin was given at 10^{-11} M. A decrease in IL-2 production was observed following deltorphin administration at 10^{-7} M revealing an unexpected biphasic modulation of lymphokine production. House et al. (1996) found that DPDPE and deltorphin administration elevated anti-CD3-induced IL-2, IL-4 and IL-6 production. Studies carried out with Jurkat T-cells transfected with the δ-opioid receptor have confirmed that δ-opioid agonist administration elevates the production of IL-2 (Hedin et al., 1997). Studies with these transfected cells suggest that the enhanced IL-2 production occurs through an opioid-induced increase in the activity of the NF-AT/AP-1 element of the IL-2 gene promoter.

Guan et al. (1997) have determined that murine thymocytes stimulated with Staphylococcal enterotoxin B (SEB) in the presence of activated macrophages exhibit significantly reduced IL-2 production following administration of U50,488H at concentrations as low as 1 nM. Similar studies have shown that IL-2 production was also decreased in a dose dependent manner in thymocytes pretreated with relatively high doses of morphine and stimulated with PHA and IL-1 (Roy et al., 1997). Analysis by RT-PCR revealed that the decrease in IL-2 production was the result of a decrease in IL-2 mRNA transcription which appeared to be due to a decrease in the level of the transcriptional activator component fos, a necessary protein for the synthesis of IL-2.

5. Effect of opioids on hematopoietic cell development

It is well established that morphine administration in vivo results in atrophy of both the thymus and the spleen (Bryant et al., 1987; Arora et al., 1990; Sei et al., 1991; Bussiere et al., 1992b; Freier and Fuchs, 1993; Fuchs and Pruett, 1993; Hilburger et al., 1997a). Several groups of investigators have found that implantation of morphine pellets subcutaneously in mice induces a rapid and profound hypoplasia (up to 70–80% reduction in thymus weight) that is sustained for approximately 3 weeks (Sei et al., 1991; Freier and Fuchs, 1993; Fuchs and Pruett, 1993). Examination of the thymic hypoplasia has revealed both significant reductions in

cellularity and perturbations in thymocyte subpopulations. A striking and consistent finding is a severe reduction in the number and percentage of CD4 + CD8 + double positive (DP) thymocytes (Sei et al., 1991; Freier and Fuchs, 1993; Fuchs and Pruett, 1993). The decrease in DP thymocytes is particularly significant because it is at the double positive stage that positive and negative selection processes occur in the thymus, ultimately determining the T-cell repertoire. Thus, it appears that morphine has the potential to influence T-cell development. Concomitant with the loss of DP cells, morphine pellet implantation resulted in alterations in the other three major thymocyte subsets, although the findings have varied among investigations. Sei and coworkers found increases in the CD4 + CD8 − , CD4−CD8 + , and CD4−CD8 − (double negative, DN) subsets, while Freier and Fuchs observed decreases in these subpopulations. Sei et al. (1991) also reported that morphine pellet implantation induced increases in the precentages of cells expressing CD3 and the IL-2 receptor. It has been shown that the effects of morphine on atrophy of the thymus and depletion of the DP cell population were mediated through classical opioid receptors, because implantation of naloxone pellets prevented the morphine-induced alterations (Sei et al., 1991; Freier and Fuchs, 1993; Fuchs and Pruett, 1993). However, it was not established whether opioid receptors on the thymocytes or in the CNS mediated these alterations. In light of the fact that morphine treatment induces a significant increase in serum corticosterone, and that glucocorticoids are capable of inducing apoptosis in DP thymocytes, it was suggested that morphine induced the depletion of DP cells via glucocorticoid-mediated apoptosis, rather than a direct influence of the opioid itself on the thymocytes. In fact, thymocytes from morphine-implanted mice exhibited increased DNA fragmentation characteristic of apoptosis (Freier and Fuchs, 1992; Fuchs and Pruett, 1993), and treatment with the glucocorticoid antagonist RU-486 or adrenalectomy blocked both the morphine-induced loss of DP cells and the increased thymocyte apoptosis (Sei et al., 1991; Fuchs and Pruett, 1993). Furthermore, direct administration of morphine to thymocytes in culture did not induce apoptosis (Sei et al., 1991).

Morphine also appears to be capable of inducing apoptosis in other populations of immune cells. Morphine has been shown to reduce numbers of macrophages and B-cells in the murine spleen, with a concomitant increase in T-cells (although the ratio of CD4 + and CD8 + cells was not altered). In contrast, in the peritoneal cavity morphine increased the number of macrphages and decreased the number of B-cells (Hilburger et al., 1997a). At the same time, nonfractionated splenocytes isolated from rats injected with morphine showed an increased percentage of cells un-

dergoing apoptosis as well as increased transcription of cathepsin-B, a gene associated with active cell death (Singhal et al., 1997). These investigators have also demonstrated that morphine increases apoptosis in a number of macrophage populations, including peritoneal macrophages from morphine-treated rats, the murine macrophage cell line J774.16, and human peripheral blood monocytes exposed to morphine in culture. These findings are noteworthy because they corroborate previous reports of morphine-mediated apoptosis of immune cells in vivo, and also indicate that in vitro morphine treatment can directly induce apoptosis in macrophages. The observation that naloxone inhibited the increased apoptosis suggests that the effect is mediated through an interaction with classical opioid receptors expressed by the macrophages. Related studies suggest that morphine may activate the apoptotic pathway in the macrophage in part through the induction of nitric oxide (Singhal et al., 1998).

Linner et al. (1995, 1996) have shown that enkephalins derived from murine thymocytes can inhibit the ConA-induced thymocyte proliferation. These investigators have suggested that thymocyte-derived enkephalins may curtail the expansion of activated T-cells in order to permit further differentiation and maturation. To pursue further this hypothesis, murine fetal thymocytes were examined for the production of endogenous enkephalin peptides. They found that the mRNA for the enkephalin precursor, proenkephalin A (PEA) is constituitively expressed in fetal thymocytes, with maximal expression on gestational day 15. Treatment of day 15 fetal thymocytes with the δ-opioid receptor antagonist, naltrindole, enhanced thymocyte proliferation, while exposure of the cells to the δ-opioid receptor agonist, deltorphin, inhibited the proliferation of day 15 fetal thymocytes. From these observations, the investigators speculate that during a specific period in gestational development, proenkephalin A is expressed to limit spontaneous proliferation of cells in the thymus, in order to allow the cells to differentiate into mature T lymphocytes (Linner et al., 1996).

There is evidence to suggest that kappa opioid receptor agonists may be capable of influencing T-cell differentiation. The expression of κ-opioid receptors by DN thymocytes as well as the immature T-cell lines R1.1 and DPK has been established by both radioactive ligand binding assays and RT-PCR (Bidlack et al., 1992; Belkowski et al., 1995a; Belkowski et al., 1995c; Guan et al., 1998). The R1.1 cell line represents a very immature double negative thymocyte with low surface expression of CD3 and CD25, and the DPK line is representative of the somewhat more mature CD3 + CD4 + CD8 + stage of thymocyte differentiation. Guan et al. (1998) observed that following stimulation with superantigen in culture for 4 days, DPK cells progressed from the double positive stage to a CD4 +

CD8INTERMEDIATE stage, with further differentiation of approximately 25% of these stimulated cells to the CD4 + CD8 − phenotype. These investigators found that the κ-opioid agonist U50,488H inhibited the super-antigen-induced differentiation of DPK cells. Approximately 60% of the DPK cells exposed to U50,488H remained CD4 + CD8 + following SEA stimulation. Cells that did undergo differentiation failed to progress beyond the CD4 + CD8INTERMEDIATE stage.

Hematopoiesis in the bone marrow also appears to be susceptible to the effects of opioids. Progenitor cells from mice treated with morphine were assessed for colony formation in culture in the presence of the growth factors macrophage-colony stimulating factor (M-CSF) or granulocyte/macrophage-colony stimulating factor (GM-CSF) (Roy et al., 1991). Cells from mice treated with morphine exhibited a 70% decrease in the number of M-CSF-induced macrophage colonies compared to placebo or control mice. There was no change in the number of GM-CSF-induced colony-forming units with cells from morphine-treated mice. Naloxone was able to inhibit the morphine-induced decrease in M-CSF-induced macrophage colonies. When bone marrow progenitor cells were treated in vitro with morphine, there was also an inhibition of M-CSF-induced colonies, but not of GM-CSF-induced colonies. Additional studies by these investigators showed that morphine treatment resulted in a dose-dependent inhibition of the proliferation of the M-CSF-dependent Bac 1.2F5 cell line. Binding studies indicated the presence of two populations of morphine-binding sites on these macrophage progenitor cells: a minor site which was naloxone-inhibitable, and a major one that appeared to be a novel opioid receptor (Roy et al., 1996).

Examination of hematopoiesis in μ-opioid receptor deficient mice revealed significantly higher absolute numbers of spleen and femoral bone marrow granulocyte-macrophage, erythroid, and multipotential progenitor cells compared to wild-type mice. Although the proliferation status and number of myeloid progenitor cells were enhanced in the μ-opioid-deficient mice, there was no alteration in mature blood cell numbers, suggesting that other regulatory interactions in leukocyte maturation may compensate at more advanced stages of cell differentiation (Tian et al., 1997). Similar analysis of δ- and κ-opioid receptor-deficient mouse strains may provide valuable additional information.

6. Opioids and resistance to infectious disease

It has long been appreciated that intravenous drug abusers have a greater incidence of infection than nonabusers (Hussey and Katz, 1950; Louria et al., 1967). The correlation of intravenous drug abuse and

HIV infection is well established (Center for Disease Control, 1996). The extent of increased infection with other organisms may not be generally appreciated. Drug abusers are reported to have a markedly increased prevalence of viral hepatitis A, B, and C, bacterial pneumonias, tuberculosis, abscesses and other soft tissue infections, CNS infections, and endocarditis (Louria et al., 1967; Reichman et al., 1979; Haverkos and Lange, 1990). Right-side endocarditis seems to have a particularly strong association with intravenous drug abuse (Reiner et al., 1976; Levine, 1991). The problem with interpretation of epidemiologic studies of infection in drug abusers is that it is difficult to determine whether the cause of the increased infection rate is due to increased exposure to infectious agents transmitted through contaminated needles and/or sexual contact, or whether contact with the drugs themselves, through suppression of immune function in the host, results in greater infectivity of the microbes. Further, little attention has been paid to the particular drug abused, the dose, and the interaction with other drugs that are likely abused with the primary drug.

There have been surprisingly few laboratory studies on the effects of opioids on infection. One of the first studies described sensitization of morphine-treated rabbits to streptococcal infection (Kraft and Leitch, 1921). In a seminal study (Tubaro et al., 1983), it was shown that single daily injections of morphine given 24–72 h prior to intravenous infection of mice with the fungus, *C. albicans*, and every other day after infection, resulted in lethality from the organism. Continuous infusion of drug by mini-pump, both pre- and post infection, similarly increased mortality, as did single daily injections of increasing doses starting at the time of inoculation. Further, animals receiving the daily increasing doses of drug had higher numbers of Candida in their kidneys. Sensitization to intraperitoneally administered Klebsiella pneumoniae also increased when morphine was injected daily, starting at the time of infection and lasting for 3 days. Interestingly, naloxone was not found to block the effects of morphine. This could be because naloxone is very rapidly metabolized in the mouse (half life of approximately 20 min (Tallarida et al., 1978)). The naloxone dosing schedule used may have been inadequate to continuosly block the effects of morphine. Based on other experiments, the increased susceptibility of the morphine-treated mice was attributed to decreased activity of phagocytic cells. Morphine depressed clearance of colloidal carbon particles from the blood, inhibited accumulation of inflammatory cells in the peritoneal cavity in response to an irritant, and depressed phagocytosis and killing of Candida by isolated macrophages and polymorphonuclear leukocytes. Unfortunately, naloxone was not tested in the in vitro studies with the phagocytic cells.

Another interesting study reported that morphine sensitized mice to infection with an avirulent strain of the parasite, Toxoplasma gondii (Chao et al., 1990). Animals given repeated subcutaneous injections of morphine every 36 h, starting 144 h before infection, and continued for 20 days after inoculation, showed marked sensitization to infection. A drug dose effect was observed. In another paradigm, mice were infected with Toxoplasma for 13 days and then given a single subcutaneous injection of morphine. Opioid-treated animals died abruptly using this regimen. However, if they were tolerized to morphine by repeated injections every 36 h, and then given a single subcutaneous injection on day 15 post infection, no animals died. This study did not explore the capacity of opioid antagonists to block sensitization to infection, but clearly demonstrated that morphine was able to render an avirulent organism lethal. Using a different paradigm, endogenous infection was reported to be enhanced by morphine. It was shown that opioid administered by subcutaneous implantation of a slow-release morphine pellet resulted in sepsis in mice, as evidenced by culture of enteric organisms from the peritoneal cavities, livers, and spleens of opioid-treated animals and their sensitization to the lethal effects of endotoxin (Hilburger et al., 1997b). Naltrexone pellets blocked sepsis and sensitization to lipopolysaccharide. The impact of morphine has also been tested in certain viral infections. In mice, herpes simplex virus-1 (HSV-1) infection was potentiated if morphine was given both before and mulitple times after infection (Panaslak et al., 1990). In a swine model of infection, it was reported that pigs made tolerant to morphine, and infected intranasally with swine herpesvirus-1 (SHV-1), had decreased neurological symptoms and mortality from the viral infection (Risdahl et al., 1993). Other animals were infected intranasally with the bacterium, Pasteurella multocida, which alone causes no disease, but in combination with SHV-1 results in pneumonia. In morphine treated animals given SHV-1 and superinfected with P. multocida, both the viral and bacterial pneumonias were more severe. Interestingly, it has also been reported that a single intracerebral injection of β-endorphin protects mice from neurological disease induced by a neurotropic strain of mouse hepatitis virus (MHV-JHM) (Gilmore and Moradzadeh, 1993). In two different studies using a murine leukemia model induced by Friend virus, morphine tolerant mice showed no alteration in susceptibility to the virus (Starec et al., 1991; Veyries et al., 1995). However, using a paradigm similar to that in the T. gondii study cited above, mice infected with the Friend virus and given morphine 2–3 weeks later, succumbed rapidly (Starec et al., 1991; Veyries et al., 1995). Naloxone was tested in one study and it blocked the morphine effects (Veyries et al., 1995).

There is great interest in the effects of drugs of abuse on the progression of HIV infection to AIDS, but the question is not subject to experimental manipulation in humans. Because the pathogenesis of HIV-1 induced disease is complex and multifactorial, host and viral factors have considerable pathophysiological significance (Fauci, 1996). Clearly, the immune response to HIV also affects the progress of the infection. The balance between HIV-1 inducing and HIV-1 suppressing host factors controls the net level of viral replication (Fauci, 1996). Two major mechanisms may potentially explain the failure of the immune response to completely eliminate HIV from the infected host. Virulence factors include formation of large pools of latently infected cells (not cleared by virus-specific cytotoxic T-cells), trapped virus particles in the follicular dendritic cell network (providing a continuous source of virus for de novo infection of migrating cells through lymphoid tissue), and changes in virus phenotype and genotype. Although virulence factors are predominantly responsible for the propagation of HIV infection over time, immunologic mechanisms generate unfavorable conditions that prevent this vigorous immune response from completely eliminating HIV. Continuous viral replication is responsible directly and/or indirectly for a number of pathogenic mechanisms that ultimately lead to the destruction of lymphoid tissue, profound immune dysfunction, and finally, progression to AIDS.

Early studies by epidemiologists found that survival following an AIDS diagnosis was shorter among HIV-infected intravenous drug users (IVDU) than other HIV-1 infected individuals, suggesting that opiate use exacerbated the disease (Rothenberg et al., 1986). However, more recent studies have suggested that progression of HIV-1 infection in IVDU as reflected by decline in CD4 + T-cell counts, is no more rapid than that reported for other risk groups (Margolick et al., 1992). It should be pointed out, however, that these epidemiological studies have limitations. IVDU may have multiple infections (causing repeated immune stimulation and recurrent HIV activation), and considerable variability in demographic and lifestyle factors that might complicate analysis of the epidemiological data (Donahoe and Vlahov, 1998). IVDUs use many different drugs, also, which have interactive effects. Such interactions also can confound epidemiological analyses. In this regard, it is important to examine the effects of chronic verses acute opiate treatment. Chronic treatment of morphine to monkeys was found to be protective against HIV replication (Donahoe et al., 1993) while withdrawal from opiates led to a transient immune depression, followed by exacerbation of disease (Chuang et al., 1993). Therefore, both viral expression and immune function differed depending on whether tolerance and dependence was disrupted. The ability of opiates to modulate the host stress response may serve

to maintain the latency states of these types of viral infections thereby reducing their pathogenic potential. In contrast, stress associated with opiate withdrawal may serve to activate the latent viruses (Donahoe and Vlahov, 1998).

Morphine has been shown to promote the growth of HIV-1 in human peripheral blood mononuclear cell cocultures (Peterson et al., 1990). In these studies, a coculture assay system was used consisting of PHA-activated peripheral blood mononuclear cells from normal donors and PBMC infected with HIV-1 from an asymptomatic patient. The increase in HIV-1 replication was shown to be reversed by naloxone, indicating that the effects involved a classical opioid receptor.

Recent in vitro studies carried out with cocultures of human brain cells and the HIV-1 latent infected promonocyte cell line, U1, showed an increase in HIV-1 expression following dynorphin A administration (Chao et al., 1995). In more recent additional studies, administration of κ-opioid agonists inhibited HIV-1 expression in acutely infected microglia cell cultures (Chao et al., 1996). Treatment of these microglial cell cultures with U50,488H resulted in a dose-dependent inhibition of the expression of the monotropic HIV-1 SF162 strain, and the effect was reversed by the κ-opioid antagonist norbinaltorphimine. The mechanism underlying the antiviral effect of κ-opioid agonists in acutely infected microglial cells in these studies is not known. However, the discrepancy between the antiviral effect of U50,488H in the microglial cultures versus the proviral effect in mixed brain cell/U1 cocultures may be explained by several factors. These would include the type of culture, the type of HIV-1 strain, and the type of HIV-1 infection (chronic vs. acute). These studies provide additional evidence that opiates may act as cofactors in the pathogenesis of HIV in IVDU. Thus, the impact of opiates on the host-parasite relationships in AIDS remains a crucial area of additional research.

7. Uncited references

Van Den Bergh et al., 1994 and Carr, 1991

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Mu-opioid receptor knockout mice show diminished food-anticipatory activity

Martien J. H. Kas,[2] Ruud van den Bos,[1] Annemarie M. Baars,[1] Marianne Lubbers,[1] Heidi M. B. Lesscher,[2] Jacquelien J. G. Hillebrand,[2] Alwin G. Schuller,[3] John E. Pintar[4] and Berry M. Spruijt[1]

[1]Ethology and Animal Welfare, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 17, NL-3584 CL Utrecht, The Netherlands
[2]Rudolf Magnus Institute of Neuroscience, Department of Pharmacology and Anatomy, University Medical Centre Utrecht, The Netherlands
[3]Millennium Pharmaceuticals Inc., Molecular Pathology, Cambridge, USA
[4]Department of Neuroscience and Cell Biology, UMNDJ-Robert Wood Johnson Medical School, Piscataway, USA

Abstract

We have previously suggested that during or prior to activation of anticipatory behaviour to a coming reward, μ-opioid receptors are activated. To test this hypothesis schedule induced food-anticipatory activity in μ-opioid receptor knockout mice was measured using running wheels. We hypothesized that μ-knockout mice show little food-anticipatory activity. In wildtype mice we observed that food-anticipatory activity increased proportional to reduced food intake levels during daily scheduled food access, and thus reflects the animal's physiological need for food. μ-Knockout mice do not adjust their schedule induced running wheel behaviour prior to and during feeding time in the same way as wildtype mice; rather than showing more running wheel activity before than during feeding, they showed an equal amount of activity before and during feeding. As food-anticipatory activity is dependent on the mesolimbic dopamine system and μ-opioid receptors regulate dopaminergic activity, these data suggest a change in the dopamine system's activity in μ-knockout mice. As we observed that μ-knockout mice tended to show a stronger locomotor activity response than wildtype mice to the indirect dopamine agonist *d*-amphetamine, it appears that the dopaminergic system *per se* is intact and sensitive to activation. We found no differences in the expression of pro-opiomelanocortin, a precursor of endogenous endorphin, in the arcuate nucleus between μ-knockout mice and wildtype mice during restricted feeding, showing that the μ-opioid receptor does not regulate endogenous endorphin levels. These data overall suggest a role for μ-opioid receptors in adapting reward related behaviour to the requirements of the environment.

Introduction

Free-living animals seem to face an almost impossible task to perform 'when and where to find the most valuable food-items to the lowest costs in an environment, which may change over short periods of time'. Yet, it has been observed that animals appear to behave relatively efficiently in such environments. For instance, they collect food-items in such a way that a minimum amount of energy is spent to get a maximum benefit (Krebs & Davies, 1993; McFarland, 1999). The behavioural and physiological systems that underlie the temporal organization of the organism's behaviour and which operate to achieve this efficiency are referred to as motivational systems (Hughes & Duncan, 1988; Jensen, 1996; Spruijt *et al.*, 2001). Motivational systems are represented in the central nervous system, among others, by the mesolimbic opioid-dopamine system (Berridge & Robinson, 1998, 2003; Spruijt *et al.*, 2001). Key words in behavioural efficiency are 'liking', 'wanting' and 'weighing/choosing' (Berridge, 1996; Berridge & Robinson, 1998, 2003; van den Bos *et al.*, 2002; van den Bos, 2004). The focus in this study is on 'wanting', which is reflected in the appetitive phase of motivated behaviour.

'Wanting' (as defined by Berridge, 1996) refers to the disposition to act upon previously 'liked' commodities. The behavioural expressions of 'wanting' are, for example, anticipatory behaviour to a coming reward in Pavlovian conditioning paradigms (rats, van den Bos *et al.*, 2003; van der Harst *et al.*, 2003a, b; Von Frijtag *et al.*, 2000; cats, van den Bos *et al.*, 2003) and lever pressing behaviour under high requirement schedules in instrumental conditioning paradigms (for a review on rats see Salamone & Correa, 2002). The behavioural expression of 'wanting' is under the control of mesolimbic dopamine systems, in particular the (core area of the) ventral striatum (Blackburn *et al.*, 1989; Berridge & Robinson, 1998, 2003; Bassareo & Di Chiara, 1999; Knutson *et al.*, 2001a, b, 2003; de la Fuente-Fernández *et al.*, 2002; Salamone & Correa, 2002; Phillips *et al.*, 1991; Peciña *et al.*, 2003).

We have previously suggested that, during or prior to the activation of anticipatory behaviour to a coming reward, the mesolimbic opioid system is activated and serves a role in overcoming, for example, fatigue, in order to achieve the goal (Spruijt *et al.*, 2001). The ventral tegmental area (VTA), which contains the cell bodies giving rise to the mesolimbic dopamine system, contains opioid receptors, among them μ-opioid receptors (Mansour *et al.*, 1995; Kitchen *et al.*, 1997). It has been shown that μ-opioid receptor activation increases the release of dopamine in the ventral striatum (Devine *et al.*, 1993; Di Chiara &

Imperato, 1988; Leone et al., 1991; Schad et al., 2002). In line with our hypothesis and these data we have obtained preliminary behavioural data showing that low dose (0.1 µg/1.0 µl) injections of the opioid antagonist naloxone into the VTA decrease anticipatory behaviour in a Pavlovian conditioning procedure (Spruijt et al., 2001).

To test further the hypothesis that activation of µ-opioid receptors plays a role in 'wanting' behaviour, schedule induced food-anticipatory activity in µ-opioid receptor knockout mice was studied. Food related anticipatory behaviour was evoked by a feeding schedule of one meal a day and measured as running wheel activity (reviewed in Mistlberger, 1994). In the first experiment, we tested whether food anticipatory behaviour reflects the animal's physiological need to eat, i.e. its motivation (Spruijt et al., 2001). We hypothesized that a decrease in mealtime duration increases the amount of running wheel activity prior to food access. Therefore, we subjected mice to different schedules of daily mealtime duration and compared the amount of wheel running activity prior to food access across these schedules. As a tool to study the role of µ-opioid receptors we used µ-opioid receptor knockout mice (Schuller et al., 1999). We hypothesized that µ-knockout mice show little, if any, food-anticipatory activity. The µ-opioid receptor is a receptor for exogenous substances, such as morphine and heroin, but also for the endogenous opiate endorphin. Endorphins are cleaved from pro-opiomelanocortin (POMC), a precursor hormone that is highly expressed in hypothalamic neurones of the arcuate nucleus. We measured the expression of POMC in the arcuate nucleus to control for differences in endorphin levels between µ-knockout mice and wildtype mice during the feeding schedules. Finally, we measured indirectly whether the mesolimbic dopamine system is sensitive to activation per se by measuring the locomotor response to injections of the indirect dopamine agonist d-amphetamine (Pijnenburg et al., 1976; Zocchi et al., 1998) We hypothesized that µ-knockout mice show a normal or even a slightly enhanced (Park et al., 2001; Tien et al., 2003) locomotor response.

Materials and methods

Subjects

Mu-knockout receptor mutant mice were created by replacing exon 1 of the MOR-1 gene with a neomycin resistant gene (Schuller et al., 1999). Mutant mice were backcrossed to a C57BL/6 background (Charles River, l'Arbresle, France) for at least nine generations resulting in a line with a standardized background. All mice were bred in our own institutes (Rudolf Magnus Institute of Neuroscience, Ethology & Welfare) from heterozygote parents. Mice were weaned at the age of 4 weeks. At weaning or one day thereafter tail tips were taken to determine the genotype.

Genotyping occurred by polymerase chain reaction on genomic DNA from tail tips. The mutant product was 700 bp, the wildtype product 525 bp; the three primers used were:
outside the mutation site
(5'-GACTTTCCTGGCTGATGCAAACAACCT-3'),
within the mutation site
(5'-CATGGTTCTGAATGCTTGCTGCGGACT-3')
and within the neomycin box
(5'-CTACCTGCCCATTCGACCACCAA-3').

All animals were housed under a 12-h light : 12-h dark cycle. Before experiments started the dark period started at 07:00 h. During experiment 1, 2 and 3 the start of the dark period was shifted to a later hour (see below). All mice were housed in iso-sex groups ($n = $ 2–4 in each group) in Macrolon type II cages with at least a tissue as enrichment in a temperature controlled room ($T = $ 21 ± 1 °Celcius;

humidity at least 50%). A radio provided background noise throughout the day. Food and water were available ad libitum. Experiments were conducted when mice were aged 3–5 months.

Experimental procedures

The Animal Ethical Committee of Utrecht University approved all experiments.

Experiment 1 Duration of food access and food-anticipatory activity

Fifteen adult female wildtype C57BL/6 mice were used for this experiment. At least 2 weeks prior to the start of the experiments mice were placed on an altered light-dark cycle. The dark period started at 12:30 h. Hereafter animals were singly housed in transparent running wheel cages (dimensions 26 × 12 × 16 cm, l × w × h). These cages contained a running wheel (diameter 14 cm; width 9 cm) and a water bottle. The bottom was covered with sawdust. Food was placed on the bottom of the cage. The mice were adapted to the running wheel for at least 1 week. The activity in the running wheel was registered by a little magnet and a counter that was activated by the magnet when it passed the counter during a revolution of the running wheel. Running wheel activity was read from the counter at 12:30 h daily during this week and expressed as daily distance moved in kilometers (up to one decimal accuracy, i.e. 100 m). During the second week the feeding regime was changed such that food was only available for 2, 3 or 4 h from 12:30 h onwards ($n = $ 5 mice per meal duration condition). Activity was scored at 10:00 h, 12:30 h, 14:30, 15:30 or 16:30 h. Throughout the experiment animals were weighed and their food intake monitored. If mice lost too much weight (> 18%) during the second week they were given some additional food at the end of the feeding period.

Experiment 2 Mu-knockout mice and food-anticipatory activity

Fifty-seven adult female wildtype, heterozygote and µ-knockout mice C57BL/6 mice were used for this experiment. The experimental conditions were as for experiment 1. We selected the food access schedule for this experiment based on experiment 1. During 4 h of food access we found relatively low levels of food anticipatory activity. As we expected a decrease in food anticipatory activity in the knockout mice the 2- or 3-h schedules were preferable. However, mice with 2 h food access showed a more rapid decline in body weight than animals with 3 h of food access (probably due to an increase in the total daily running during food restriction for the 2 h condition). Therefore, the 3 h schedule was used in this experiment.

Experiment 3 POMC expression

Adult female wildtype ($n = $ 5) and µ-knockout ($n = $ 5) C57BL/6 mice were used for this experiment. Brains were removed 2 h prior to the second day of the scheduled 3-h food access. They were then frozen in cold isopentane (−30 °C for 20 s). Pre-treated 16-µm cryostat sections from mouse hypothalamus were hybridized with ^{33}P-labelled antisense RNA probes according to van der Kraan et al. (1998). A 350 bp rat POMC cDNA fragment (from bp +97 to +447 relative to transcription initiation) was used as the template for synthesis of the RNA probe for in situ hybridization. POMC mRNA expression levels in the arcuate nucleus were quantified using MCID-M5 (Imaging Research, Ontario, Canada).

Experiment 4 d-amphetamine induced activity

Thirty-five adult female wildtype, heterozygote and µ-knockout C57BL6 mice were used for this experiment. Animals were kept on

their initial light-dark cycle period for this experiment. All testing was carried out during the dark period between 10:00 h and 16:00 h. Mice were placed in transparent test cages (dimensions 62 × 26 × 33 cm, l × w × h). The test cage contained saw dust (Lignocel type 3/4; cf. home cage) as bedding material. Test cages were cleaned between sessions for different mice. Mice were allowed to habituate for 30 min. Hereafter, they received subcutaneous injections of vehicle (0.9% NaCl) or d-amphetamine (d-amphetamine sulphate dissolved in 0.9% NaCl). A dose of 4 mg/kg (0.5 mL/100 g bodyweight) was used, which has been shown to induce a reliable increase in activity in CB57BL/6 mice (Zocchi *et al.*, 1998). All d-amphetamine solutions were prepared beforehand at the dispensary of the Veterinary Faculty and frozen until use. The behaviour of the mice was recorded for at least 80 min. Throughout the experiment the behaviour of the mice was recorded by Ethovision (Noldus Information Technology BV, Wageningen, The Netherlands) on a PC at a sample rate of five samples per second. Littermates were tested simultaneously (four maximum).

Dependent variables

Experiment 1 and 2

Daily weight and food-intake of the mice were recorded. During the second week of experiments, daily running wheel activity was scored, i.e. the activity from 12:30 h on one day to 12:30 h on the next. Day 0 was defined as the 24 h prior to the first restricted feeding period. Days 1–4 were the four 24 h periods during which food restriction occurred. The running wheel activity prior to feeding was scored from 10:00 to 12.30 h, i.e. during a 2.5-h period. The running wheel activity during feeding was scored from 12:30 to 14:30, 12:30 to 15:30 or 12:30 to 16:30 h, depending on the specific test condition. All data are in kilometres, accurate to 100 m. Group scores are expressed as mean ± standard error of the means (SEM) unless otherwise indicated.

Experiment 3

The dependent variable was POMC mRNA expression (c.p.m.). Group scores are expressed as mean ± SEM unless otherwise indicated.

Experiment 4

The dependent variable was the distance moved (in meters) extracted from the Ethovision files. All group data are expressed as mean ± - SEM unless otherwise indicated.

Statistics

The data from the different experiments were analysed by paired t-tests, and one-way, two-way or three-way analysis of variance (ANOVA) depending upon the specific dataset using SPSS version 9.0 for Windows. Factors are indicated in the Results section. *Posthoc* testing was carried out whenever appropriate, and is indicated in the Results section. Significance was set at $P \leq 0.05$; $0.05 < P \leq 0.10$ was taken to indicate a trend, whereas $P > 0.10$ was taken as not significant (NS). Unless otherwise indicated all statistics are two-tailed.

Results

Experiment 1

Mice with daily scheduled food access 2 h per day (in the first 2 h of the dark phase, their habitual activity phase) ate significantly less than mice with 4 h of food access per day [mean ± SEM 30.6 ± 2.0% vs. 57.6 ± 2.6% of their *ad libitum* daily food intake; one-way ANOVA (including 3 h) $F_{2,12} = 31.098$, $P \leq 0.001$, *posthoc* Student–Newman–Keuls test] and exhibited a significant increase in wheel running activity in the hours prior to food access (Fig. 1A). Mice with three (34.9 ± 3.1%) or 2 h of food access had similar amounts of food intake (statistics, as mentioned see above) and expressed similar levels of anticipatory behaviour prior to food access. Relative food intake during restricted food access was highly correlated with the amount of wheel running prior to food access (Fig. 1B; $r = -0.69$, $n = 15$, $P \leq 0.005$).

Experiment 2

Seven mice were discarded from the final analysis in the running wheel experiment; three (one individual of each genotype) because of technical problems with the running wheels, four (two wildtype mice, one heterozygote mouse and one μ-knockout mouse) because of not showing substantial running wheel activity (< 25 km total activity over 2 weeks). This left $n = 16$ wildtype (WT), $n = 16$ heterozygote mice (HT) and $n = 18$ μ-opioid receptor knockout mice (KO) mice for final data analysis.

Figure 2A shows the total daily running wheel activity for the different genotypes over the course of five days. The data show that no difference was present between the different genotypes, neither over the course of the experiment [two-way ANOVA, genotype, day (repeated measure): genotype × day $F_{8,188} = 1.062$, n.s.] nor independent of day (genotype $F_{2,47} = 0.046$, n.s.). All mice showed a decrease over the course of the experiment ($F_{4,188} = 34.513$, $P \leq 0.001$). However, a clear effect was found in the 2.5 h before feeding (Fig. 2B), i.e. although KO mice showed an increase in running wheel activity prior to food access during scheduled feeding this increase in activity was clearly lower than that of the HT and WT mice [two-way ANOVA, genotype, day (repeated measure), genotype × day $F_{8,184} = 2.550$, $P \leq 0.012$; genotype $F_{2,46} = 3.837$, $P \leq 0.029$]. The opposite was found for the running wheel activity during the 3-h feeding period, i.e. KO mice tended to show a lower decrease in running wheel activity than WT and HT mice [two-way ANOVA, genotype, day (repeated measure), day $F_{4,188} = 69.590$, $P \leq 0.001$; genotype × day $F_{8,188} = 1.668$, n.s.; genotype $F_{2,47} = 5.668$, $P \leq 0.006$]. HT mice especially, showed a strong decrease in running wheel activity. When the two periods were compared as running wheel activity per hour (Fig. 2D), it turned out that KO mice did not adjust their running wheel activity to the same extent as WT and HT mice: whereas WT and HT mice showed a clear shift over the days from more running wheel activity during feeding to more running wheel activity prior to feeding, KO mice showed only a partial shift, i.e. from more running wheel activity during feeding to similar running wheel activity prior and during feeding [three-way ANOVA, genotype, day (repeated), prior–during feeding (repeated): genotype × day × prior–during feeding: $F_{8,184} = 2.318$, $P \leq 0.022$].

No differences were observed between the different genotypes for either body weight or food intake during the experiment (Fig. 2E and F). All genotypes showed an equal decrease in body weight following food restriction, on average 82% of the free-feeding weight [two-way ANOVA, genotype, day (repeated measure): genotype × day $F_{8,188} = 0.388$, n.s.; day $F_{4,192} = 669.486$, $P \leq 0.001$]. With respect to food intake mice rapidly adjusted their food intake during the food restriction period to consuming more food following the first restriction day. It appeared that during the first day WT mice showed somewhat less consumption than HT and KO mice but caught up after this initial day [two-way ANOVA, genotype, day (repeated measure):
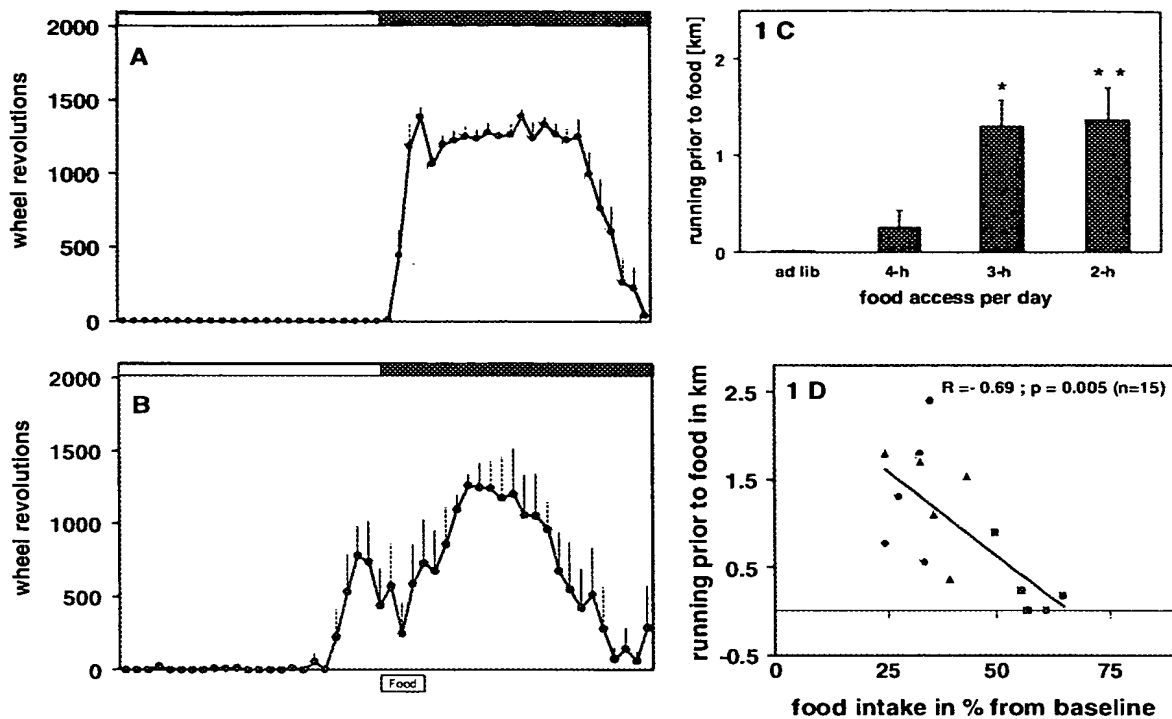
FIG. 1. (A) Nocturnal rodents with voluntary access to running wheels and ad libitum access to food exhibit a circadian wheel running rhythm with high levels during the dark phase, their habitual activity phase, and almost no running during the 12-h light phase of the 24-h light-dark cycle. (B) Daily scheduled food availability results in a re-distribution of wheel running activity over the 24-h day. Scheduled food access is preceded with high levels of behavioural activity in the hours prior to food access (box with food below the x-axis indicate hours of food access). (C) Averaged (3 days) running wheel activity prior to food access of mice (2.5 h; $n = 5$ mice per group) subjected to food restriction and 2, 3 or 4 h of food access. Activity prior to food access was compared to baseline ad libitum activity using paired t-tests. As no differences were found between different baseline conditions, the mean of baseline activity is shown for all 15 mice, paired t-tests were run however, on the original group data. Values were 4 h, $t = 1.496$, n.s.; 3 h, $t = 4.043$, $P \le 0.02$; 2 h, $t = 4.798$, $P \le 0.01$; *$P \le 0.05$, **$P \le 0.01$. (D) Pearson product-moment correlation of averaged (3 days) running wheel activity prior to food access and averaged (3 days) relative food intake of mice. Different symbols indicate different groups; ■ 4 h of food access; ▲ 3 h of food access; ● 2 h of food access.

genotype × day: $F_{6,138} = 2.564$, $P \le 0.022$; day: $F_{3,138} = 291.717$, $P \le 0.001$]. During ad libitum feeding conditions no differences were found between wildtype, heterozygote and μ-knockout mice in food intake, 4.73 ± 0.16, 4.74 ± 0.23 and 4.49 ± 0.24 g (mean ± SEM), respectively (one-way ANOVA, $F_{2,47} = 0.457$; n.s.).

Experiment 3

Figure 3A–C shows the expression of POMC in the arcuate nucleus of μ-knockout mice and wildtype mice during scheduled feeding. No differences were observed between μ-knockout mice and wildtype mice ($t = 1.553$, d.f. $= 8$, n.s.).

Experiment 4

In Fig. 4A–C it can be seen that the 4 mg/kg dose of d-amphetamine enhanced locomotor activity compared to vehicle [three-way ANOVA, genotype, drug, time bin (repeated measure): drug × time bin $F_{15,435} = 6.360$, $P \le 0.001$] in each genotype (genotype × drug × time bin $F_{30,435} = 0.544$ n.s.). Based on the three-way ANOVA, the increase in locomotor activity following d-amphetamine did not differ between genotypes. When comparing the locomotor response to d-amphetamine within genotypes, it should be noted that the stimulant effect was most prominent in the HT and KO mice: drug × time bin

for WT ($F_{15,120} = 1.505$, n.s.), HT ($F_{15,180} = 3.662$, $P \le 0.001$) and KO ($F_{15,135} = 2.624$, $P \le 0.002$).

Discussion

The present study revealed that running wheel activity prior to scheduled food access is related to the animal's motivation for food. Furthermore, mice lacking the expression of the μ-opioid receptor, i.e. μ-knockout mice (Schuller et al., 1999), do not adjust their schedule induced running wheel behaviour prior and during feeding time in the same way as wildtype mice. Rather than showing more running wheel activity before than during feeding, they show equal activity before and during feeding. Whilst all genotypes responded to d-amphetamine with increased locomotor activity, μ-knockout mice tend to have a stronger locomotor response to d-amphetamine than wildtype mice. Finally, μ-knockout mice do have a normal expression of POMC, the precursor of endogenous endorphin.

Schedule induced activity

As food intake during restricted food access is highly correlated with the amount of wheel running prior to food access, these data indicate that the intensity of food anticipatory activity highly reflects the animal's physiological need to eat, i.e. its motivation. Under
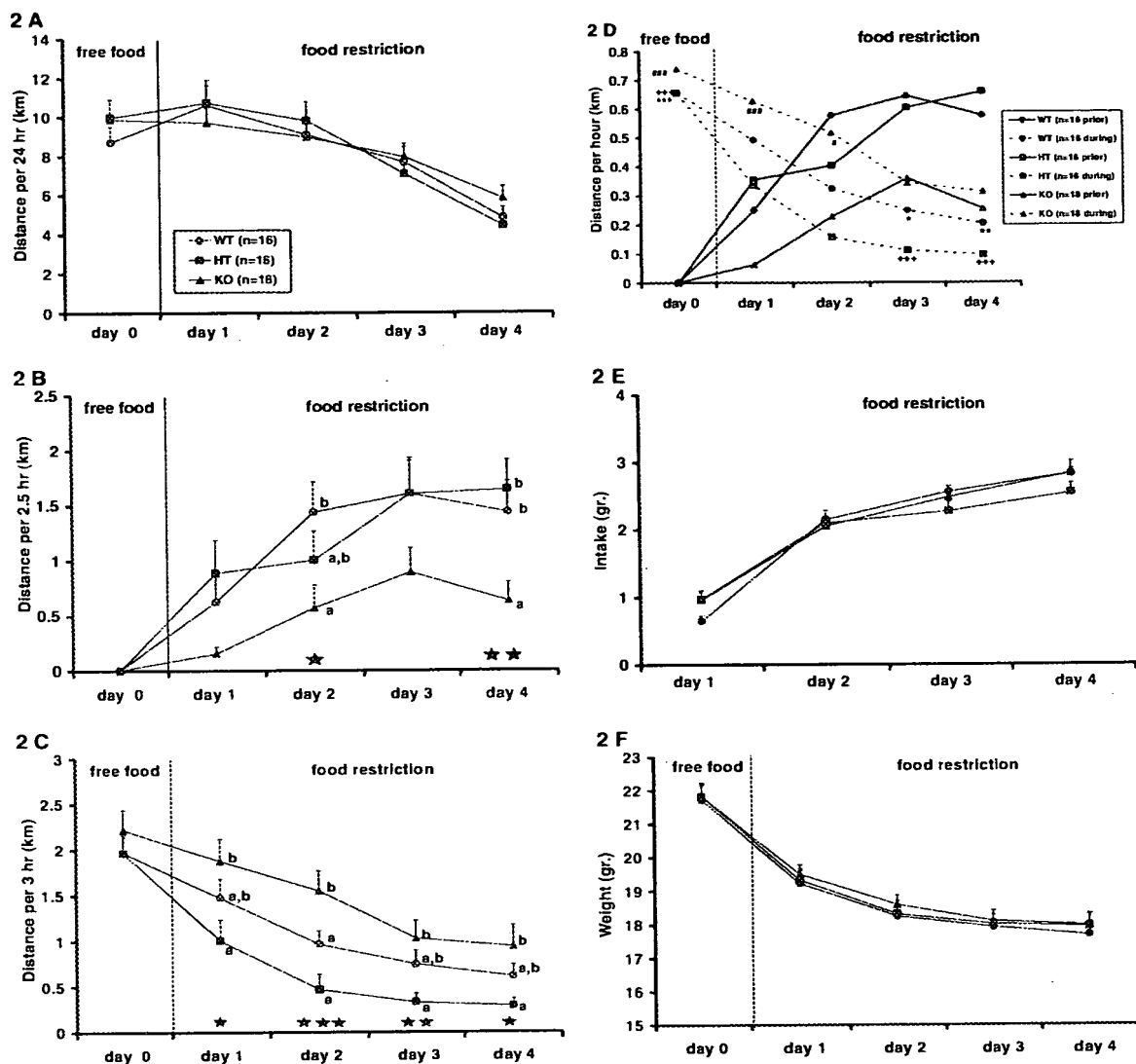
FIG. 2. (A) Mean daily running wheel activity during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. (B) Mean 2.5 h running wheel activity prior to food access during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. $*P \leq 0.05$, $**P \leq 0.01$ [one-way ANOVA; groups with the same characters are not different from one another (*posthoc* Student–Newman–Keuls)]. (C) Mean 3-h running wheel activity during food access during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ [one-way ANOVA; groups with the same characters are not different from one another (*posthoc* Student–Newman–Keuls)]. (D) Mean hourly running wheel activity prior to and during food access during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. SEMs are omitted for clarity; #, + , $*P \leq 0.05$, ##, ++ , $**P \leq 0.01$, ###, +++ , $***P \leq 0.001$ (paired *t*-test, during vs. prior; for KO, HT and WT, respectively). (E) Mean food intake during food access restricted feeding conditions, i.e. 3 h of daily food access (day1–4) for wildtype, heterozygote and μ-knockout mice. (F) Mean body-weight during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (day1–4) for wildtype, heterozygote and μ-knockout mice.

laboratory conditions it seems paradoxical that animals become very active during times of low food availability, as food will become available without having to work for it. However, in view of behavioural efficiency, the increased expression of · anticipatory behaviour during reduced food availability likely mirrors an important motivational behaviour in the survival of an organism in its natural environment. For example, in times of restricted and timely available food access, an organism requires to establish a proper balance

between its reduced energy intake and daily energy expenditure. The observed increased expression of anticipatory behaviour may reflect exploratory behaviour in the search for food.

μ-knockout mice and anticipatory activity

Before discussing the differences around feeding time between μ-knockout mice and wildtype mice it is worth noting that the effects
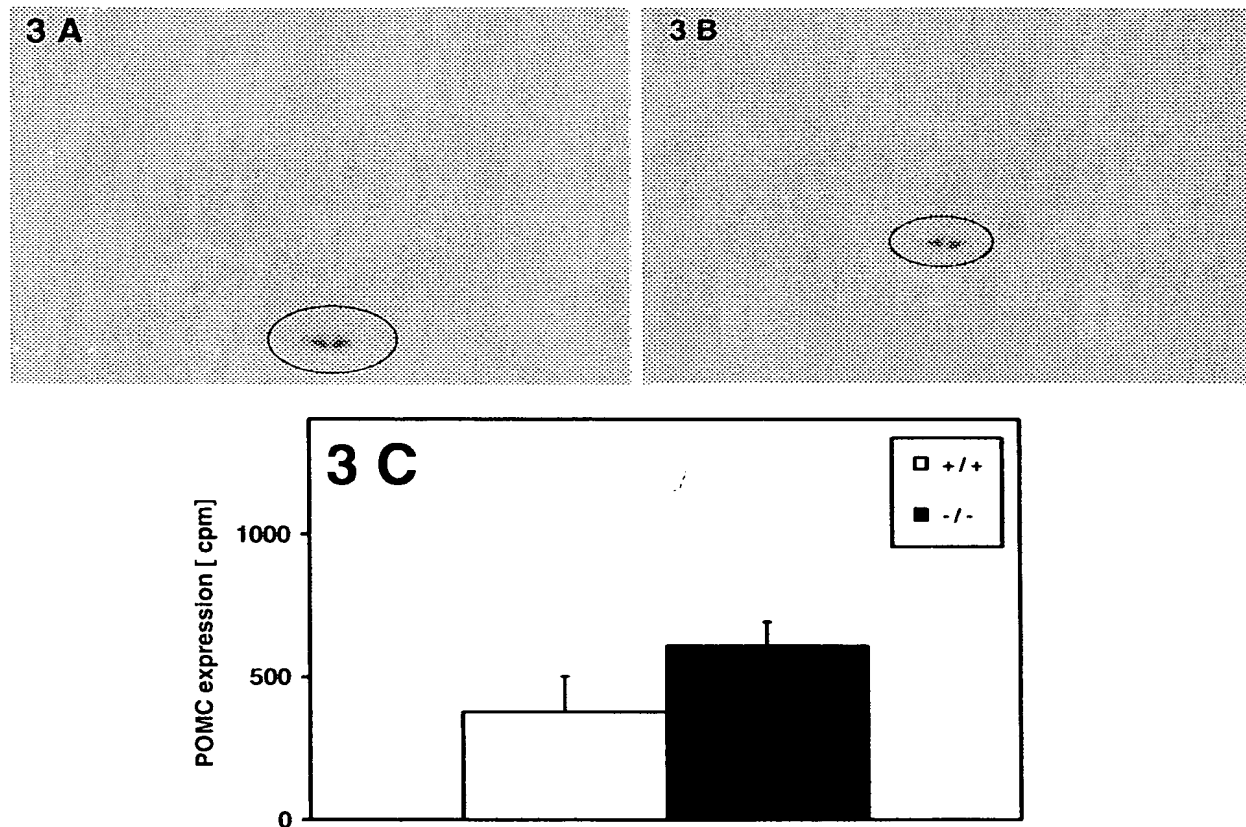
FIG. 3. (A) POMC expression in the arcuate nucleus of the hypothalamus (encircled) of wildtype mice prior to food access. (B) POMC expression in the arcuate nucleus of the hypothalamus (encircled) of μ-knockout mice prior to food access. (C) Mean POMC expression in the arcuate nucleus of wildtype and μ-knockout mice prior to food access.

observed during this period are not due to differences in general daily running wheel activity, weight or food intake changes *per se*, as no effects were observed on any of these parameters. The data show that, as in *ad libitum* fed mutant mice (Zhou *et al.*, 2002), POMC gene expression is not affected in μ-receptor knock mice as a consequence of scheduled food access. These data show that the endogenous μ-opioid receptor affects food anticipatory behaviour, but does not impair endogenous endorphin expression levels during *ad libitum* and scheduled daily food access. Moreover, no differences were observed between μ-knockout mice and wildtype mice with respect to blood glucose levels prior to feeding (unpublished data) ruling out metabolic differences between genotypes underlying the differences in running wheel activity.

The change from free food to restricted food induced a clear change in the running wheel behaviour of the wildtype mice. Whereas no running wheel activity was seen in the 2.5 h preceding the onset of the dark period under free food conditions, running wheel activity began to occur in this period when food was only available during the first 3 h of the dark period. These results are in line with those observed in other studies on C57BL/6 mice (Abe *et al.*, 1989; Marchant & Mistlberger, 1997) and in general, on food-anticipatory activity under restricted feeding schedules (Mistlberger, 1994). In wildtype mice a strong decrease occurred in running wheel activity during the feeding hours, concurrently with the increase of running wheel activity before the feeding hours. Under free food conditions mice showed far more

activity during the first hours of the dark period than in the hours before the onset of the dark period, whereas under restricted food conditions mice showed far more activity before the onset of the dark period than during the first hours of the dark period when food was available. Although several models exist to account for the changes seen in this running wheel behaviour, no model until now fully explains the changes in behaviour (see, e.g. Mistlberger & Marchant, 1995; Mistlberger *et al.*, 2003). It has been shown that the development of food-anticipatory activity is neither dependent on the suprachiasmatic nucleus (Marchant & Mistlberger, 1997) nor the lateral hypothalamus (Mistlberger *et al.*, 2003) but involves the hippocampus and cortical areas (Wakamatsu *et al.*, 2001). Whatever the underlying mechanism, it is clear that a form of learning takes place. As we observed that during four days of the schedule the MOR-deficient mice developed food-anticipatory behaviour but at a lower overall level than wildtype mice and heterozygote mice, the data therefore suggest that μ-opioid receptors are not necessary for the development of food-anticipatory activity, i.e. for this learning process *per se*. Consistent with this, the decrease in running wheel activity during the first hours of the dark period, i.e. during the period that food was present, also occurred in the μ-knockout mice, however, halted to the point that an equal amount of activity was seen prior and during feeding.

In so far as food-anticipatory activity may be considered identical to anticipatory activity under a Pavlovian conditioning schedule, or

appetitive behaviour prior to consummatory behaviour (Spruijt *et al.*, 2001) regulated by the mesolimbic dopamine system (Blackburn *et al.*, 1989; Phillips *et al.*, 1991; Berridge & Robinson, 1998; de la Fuente-Fernández *et al.*, 2002), these data therefore suggest that μ-opioid receptors in the VTA, which the μ-knockout mice lack (Kitchen *et al.*, 1997), are not necessary for the development of anticipatory behaviour *per se*. The overall change in schedule induced behaviour resembles that obtained by Parkinson *et al.* (1999) for cue-induced (CS) magazine visit behaviour observed in animals with lesions of the core-region of the ventral striatum: no change in total number of magazine visits, but a clear failure to specifically increase magazine visits while the CS announces the arrival of a reward resulting in similar activity before and after the reward (US). Both running wheel activity (Vargas-Pérez *et al.*, 2003) and food restriction (Cadoni *et al.*, 2003) have a profound effect on the core area of the ventral striatum, which is involved in anticipatory behaviour (Bassareo & Di Chiara, 1999). The present data would thus suggest that μ-opioid receptors in the VTA driving dopaminergic activity in the core area of the ventral striatum play a role in adapting reward related behaviour to the requirements of the environment; when it is opportune to behave in relation to the arrival of rewards, such as food (cf. Schultz, 2000; Phillips *et al.*, 2003). In so far as food anticipatory activity and behavioural sensitization under dopaminergic stimulant drugs are dependent on the core area of the ventral striatum (Cadoni *et al.*, 2003) the data of the present study fit in with the observation that μ-knockout mice do show a cocaine induced sensitized motor response *per se*, but at a lower overall level than wildtype mice (Yoo *et al.*, 2003).

Although we conclude that μ-knockout mice have an impaired regulation of their anticipatory behaviour to rewarding stimuli, it may be suggested that the hedonic valuation of rewarding stimuli is reduced in these mice, which may explain the decrease in anticipatory activity. However, we observed in the present study that food intake levels between wildtype mice and μ-knockout mice were not different. If consumption is taken as a measure for hedonic valuation, then no differences exist between wildtype and μ-knockout mice for hedonic valuation. Whilst this may have been caused by the limited amount of time to eat, we have also observed that μ-knockout mice have a similar increased preference for sucrose (5% sucrose vs. water), another measure for hedonic valuation, compared to wildtype mice in a two-bottle free-choice paradigm; sucrose preference ratios after 3 days 0.95 ± 0.01 (wildtype mice) vs. 0.96 ± 0.01 (μ-knockout mice). Overall, these data are in line with a recent study in mice lacking endogenous β-endorphins (the natural ligand for μ-opioid receptors; Hayward *et al.*, 2002), and suggest that the hedonic aspects of food consumption were not reduced in μ-knockout mice. Furthermore, it should be noted that the appraisal of food not only relies on reward systems as discussed here but also on food regulatory mechanisms in the hypothalamus that tend to dominate under conditions of deprivation or metabolic stress, such as in the present study, and thus over-rule the hedonic effects of endorphins (Hayward *et al.*, 2002). Unless more sensitive measures for the hedonic aspects of food consumption become available that prove differently, the data indicate that μ-opioid receptor signalling is at least involved in the appetitive phase of food motivated behaviours under the present conditions.

d-amphetamine induced activity

Overall μ-knockout mice tended to show a stronger *d*-amphetamine induced locomotor response than wildtype mice. This seems to be in line with the observation that μ-knockout mice have supersensitive dopamine receptors compared to wildtype mice (Park *et al.*, 2001;
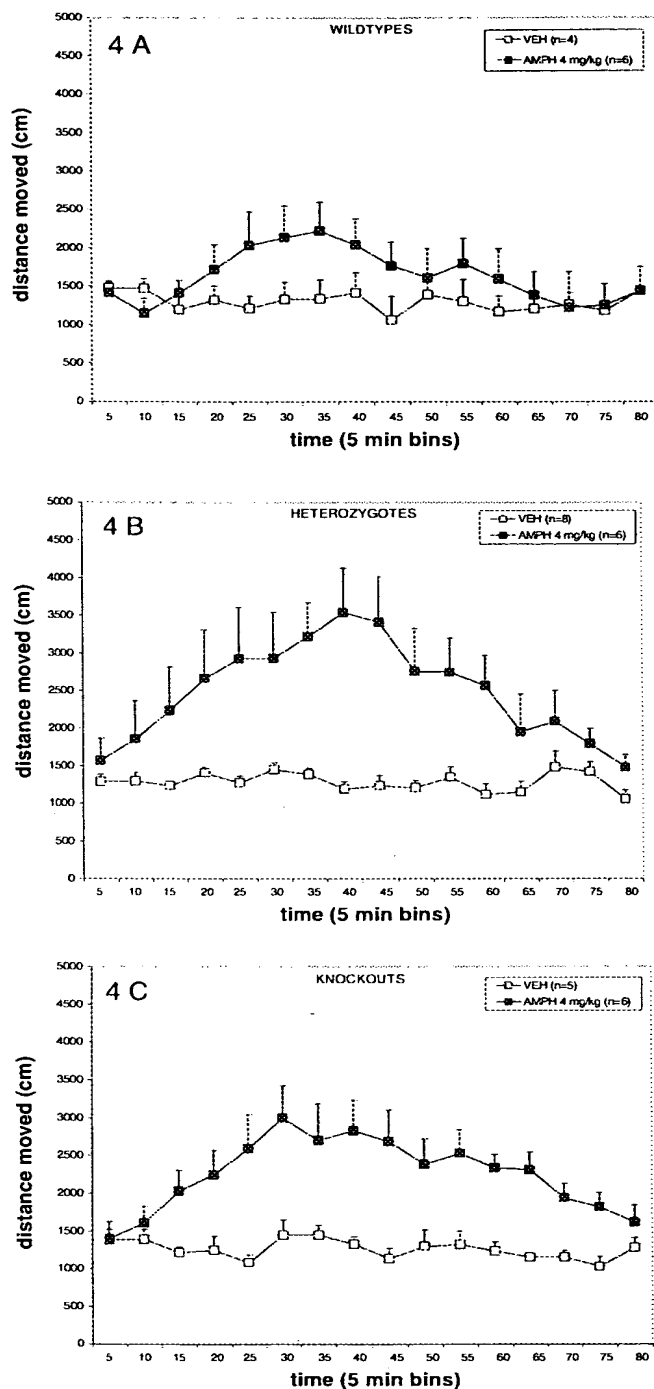
Fig. 4. (A) Mean distance moved after injection of vehicle or *d*-amphetamine (4 mg/kg) in wildtype mice during 80 min, expressed as 5-min bins. (B) Mean distance moved after injection of vehicle or *d*-amphetamine (4 mg/kg) in heterozygote mice during 80 min, expressed as 5-min bins. (C) Mean distance moved after injection of vehicle or *d*-amphetamine (4 mg/kg) in μ-knockout mice during 80 min, expressed as 5-min bins.

Tien *et al.*, 2003). The dose of *d*-amphetamine should have produced a stronger response in wildtype mice (Zocchi *et al.*, 1998). However, the present mice were tested without any prior manipulation, whereas those of Zocchi *et al.* (1998) were isolated for 48 h before testing. Short-term isolation may enhance the responsiveness to *d*-amphetamine (Kokkinidis & MacNeill, 1982; Cabib & Puglisi-Allegra, 1996). Whatever the explanation for this discrepancy may be, the differences between μ-knockout mice and wildtype mice on schedule induced behaviour are not due to a dysfunctioning dopamine system *per se*, but rather the way the mesolimbic dopamine system is activated under scheduled feeding.

Conclusion

The present data strongly suggest that μ-opioid receptors play a crucial role in adapting reward related behaviour to the requirements of the environment, when it is opportune to behave in relation to the arrival of rewards, such as food.

Abbreviations

AbbreviationsHT, heterozygous; KO, knockout; n.s., not significant; POMC, pro-opiomelanocortin; VTA, ventral tegmental area; WT, wildtype.

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Review

Opioid peptides and the control of human ingestive behaviour

Martin R. Yeomans*, Richard W. Gray

Laboratory of Experimental Psychology, University of Sussex, Brighton, Sussex BN1 9QG, UK

Abstract

A variety of evidence suggests that endogenous opioid peptides play a role in the short-term control of eating. More recently, opioid receptor antagonists like naltrexone have been approved as a treatment for alcohol dependence. Here we review the evidence for a role of opioid peptides in both normal and abnormal eating and drinking behaviours and in particular try to identify the nature of the role of opioids in these behaviours. Particular attention is paid to the idea that opioid reward processes may be involved both in the short-term control of eating and hedonic aspects of alcohol consumption, and parallels are drawn between the effects of opiate antagonists on food pleasantness and the experience of drinking alcohol. The review also explores the extent to which data from studies using opiate antagonists and agonists provide evidence for a direct role of endogenous opioids in the control of ingestive behaviour, or alternatively whether these data may be better explained through non-specific effects such as the nausea commonly reported following administration of opiate antagonists. The review concludes that the present data suggests a single opioid mechanism is unlikely to explain all aspects of ingestive behaviour, but also concludes that opioid-mediated reward mechanisms play an important control in hedonic aspects of ingestion. The review also highlights the need for further empirical work in order to elucidate further the role of opioid peptides in human ingestive behaviour.
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Keywords: Opioid peptides; Opiate receptors; Feeding; Drinking; Alcohol; Reward; Hedonics

Contents

* Corresponding author. Tel.: +44-1273-678617; fax: +44-1273-678058.

E-mail address: martin@biols.sussex.ac.uk (M.R. Yeomans).

Since their discovery in the 1970s, evidence has accumulated that opioid peptides play a role in the control of human ingestive behaviour. This review re-evaluates that role in the light of the increased use of drugs which selectively block

opioid receptors (opioid receptor antagonists) in the treatment of alcohol abuse and to a lesser extent in binge eating, and considers whether the effects of opioid antagonists as a preventive measure in alcohol abuse and as a modulator of eating behaviour reflect a common action on the endogenous opioid system. The review concentrates on the effects of opioid antagonists on human ingestive behaviours starting with eating and normal drinking, and then explores effects in relation to alcohol consumption, relapse prevention and abuse. Studies of effects of opioid agonists are also reviewed, but data here are much more limited due primarily to the practical and ethical difficulties of conducting experimental work with these drugs in humans. The review also highlights areas where knowledge is limited and further research is warranted. The discussion centres on research with humans, but brief discussions of the outcome of similar studies in animals are included where these clarify the outcome of human studies.

1. Opioids and eating behaviour

The first evidence that opioid peptides may play a role in the short-term regulation of feeding came from a study of the effect of the general opioid receptor antagonist on eating in the rat [1]. Naloxone caused a significant reduction in short-term food intake, an effect which has since been replicated in a very wide range of animals including species as diverse as birds, most rodents and even tigers [2]. More detailed analysis of the effects of opioid antagonists on feeding in animals has lead to suggestions that opioids play a particular role in hedonic aspects of eating and orosensory

reward processes [3–5]. Evidence to support that hypothesis included the finding that naloxone reduced food intake with palatable diets [6–8], was more potent at reducing intake of a sucrose-rich than cornstarch-rich diet [9] and had a greater effect on drinking palatable solutions than on drinking water [10–13]. Naloxone also selectively reduced intake of sweet solutions in sham-fed rats [14], and continual exposure to preferred foods enhanced the anorectic potential of naloxone [15–17]. Overall, these data make a strong case in the rat for opioid involvement in mediating the enhancement of feeding through food palatability, and the present review examines the extent to which a similar conclusion can be reached from studies in humans.

Pharmacological studies of the role of opioids in human appetite control have been limited mainly to investigations using the general opiate receptor antagonists naloxone (administered intravenously), naltrexone and nalmefene (both administered orally). Although this research has been reviewed elsewhere [18,19], here we re-examine these studies in order to evaluate more fully the likely mechanisms which underlie the effects of these drugs on normal and disordered eating, normal drinking and alcohol abuse.

2. Effects of opioid receptor antagonists and agonists on short-term food intake in normal-weight subjects

There have been 11 controlled studies of the effects of the general opioid antagonists naloxone [20–24], naltrexone [25–30] and nalmefene [31,32] on short-term food intake in normal-weight humans (Table 1), and all but one [28]

Table 1
Effects of opiate antagonists on food intake, rated hunger and food pleasantness in normal subjects

Reference	Subjects	Drug, route and dose	Effects on appetite		
			Food intake	Hunger at start of meal	Rated food pleasantness
[20]	8 men	Naloxone, IV 18 mg in 4 h	25% decrease	No effect	Not tested
[21]	12 men	Naloxone, IV 0.8 and 1.6 mg	25% decrease with 1.6 mg	No effect	Not tested
[22]	7 men	Naloxone IV 2 mg/kg	28% decrease	No effect	Not tested
[25]	6 men + 2 women	Naltrexone, oral, 60 mg	–	No effect	Decreased
[26]	11 men + 3 women	Naltrexone, oral 25 mg	–	No effect	Potentiated alliesthesia
[31]	20 men	Nalmefene, oral 2.5 mg	21% decrease	No effect	Not tested
[27]	18 men	Naltrexone, oral 50 mg	19% decrease	No effect	Decreased
[28]	12 men, 14 men	Naltrexone, oral 50 mg	No effect, 24% increase in ice-cream intake	No effect	No effect
[32]	24 men	Nalmefene, oral 2.5 mg	22% decrease	No effect	Decreased
[23]	15 women	Naloxone, IV 6 mg + 0.1 mg/kg 2.5 h	17% decrease	No effect	Decreased
		Butorphanol, IV 1 μg/kg	No effect	No effect	Marginally increased
[29]	16 men	Naltrexone, oral 50 mg	14% decrease	No effect	Decreased
[30]	20 men	Naltrexone, oral 50 mg	23% decrease	No effect	Decreased
[64]	18 women	Naltrexone, oral 50 mg	Not tested	Not tested	Decreased
[24]	10 men and 14 women	Naloxone, IV	11–13% decrease	No effect	Not tested

reported significant reductions in intake. Moreover, despite the variety of eating tests, drugs and test situations used, the extent of intake reduction was remarkably consistent, with all nine successful studies reporting reductions in the range of 11–29% (mean reduction 21%, weighted for sample size). This narrow range of outcomes itself implies a specific and consistent effect on eating control. If the administration of a specific receptor antagonist modifies a behavioural response, then the implication is that the antagonist is blocking the normal function of an endogenous agonist. The specific receptor binding profile of the antagonist can then be used to provide clues about the likely endogenous ligand. Accordingly, research in animals has established that μ, σ and κ receptors are all implicated in the short-term control of feeding [33,34]. However, human studies with opiate receptor antagonists have so far lacked the pharmacological sophistication to make inferences about underlying endogenous ligands since the receptor-specific antagonists used in animals are yet to be approved for human use.

Before evaluating the potential roles of opioids in the control of ingestive behaviour, it is important to evaluate the alternative possibility that reduced eating following opioid antagonist administration reflects a non-specific consequence of drug-related side effects, since this possibility has been raised in several of the studies using opioid receptor antagonists. For example, some studies reported nausea as a side effect of administration of naltrexone in studies of eating [27,29], and more recently nausea has been reported as a significant side effect of naltrexone when used as a treatment for alcohol dependence [35]. If people feel nauseous after administration of naltrexone, then this could cause a non-specific reduction in eating. However, in laboratory studies of eating, naltrexone-induced nausea was relatively rare, with only 19% of subjects in our most recent studies reporting nausea after naltrexone, compared with 9% following placebo [29,30]. Further analysis of intake data from studies with naltrexone also suggested that rated nausea was a poor predictor of changes in intake [27,29]. Other studies using naltrexone did not find evidence of drug-induced nausea [24], and nausea was not reported in the studies with naloxone [21, 23] or nalmefene [31,32]. Together, these data strongly imply that effects on intake cannot be explained away as an indirect consequence of drug-induced nausea. Other non-specific explanations are also unlikely, since other side effects of the drugs in these studies have been limited. Overall, careful scrutiny of these studies of short-term effects of opioid antagonists on eating suggest that the reductions in food intake reflect a direct effect on appetite control rather than an indirect, non-specific effect.

No study has examined the effects of opioid agonists such as morphine on short-term eating in humans, but studies in animals generally show enhanced feeding after opioid agonist administration [33,36–40,41]. However, there have been exceptions, notably in studies involving selective κ receptor agonists, which can also reduce feeding, particularly with more palatable diets [42,43]. In humans, there have been

a limited number of reports of the effects of mixed agonist/antagonist drugs on eating, but the results have also been inconsistent. Although in one human study butorphanol doubled caloric intake in the 2 h post-injection relative to placebo, and naloxone partially reversed this effect [44], the only other published study with butorphanol in normal subjects found no effects on food intake and only a marginal (non-significant) increase in taste preference [23]. In contrast, butorphanol reliably induces feeding in rats [45,46], and is generally more effective that other opioid agonists [47]. Butorphanol-induced feeding is reversed by selective μ [48], but not κ [49] receptor antagonists, and the specific brain sites underlying butorphanol-induced feeding in rats have been characterised [50]. More studies with this class of compounds are therefore needed in humans to assess more clearly the potential stimulation of appetite by opioid agonists.

3. Potential mechanisms for opioid actions in the control of normal eating

The control of short-term food intake is highly complex, and involves multiple underlying motivational systems [51]. Opioids could potentially modify any or several of these underlying controls, and disentangling any specific effect requires detailed evaluation. Here, we explore the evidence for and against a role of opioids in different aspects of eating motivation.

4. Opioids and hunger?

Modulation of hunger, defined in terms of the sensations associated with the progressive utilisation of nutrients in the absence of feeding, would be expected to alter short-term food intake, and there is a large literature showing that short-term manipulations of nutrient requirements using the preloading technique can modify subsequent eating [52–59, 60]. If opioid antagonism caused a general suppression of appetite, then the effects of opioid antagonists should be similar to the effects of energy preloads. However, rated hunger at the start of the test meals did not differ between antagonist and placebo conditions in any of the studies published to date (Table 1), in contrast to the effects of other manipulations of appetite such as caloric preloading [58,59]. Naloxone also failed to reduce rated hunger which had been elevated in response to 2-deoxy-D-glucose-induced glucoprivic stress [20]. Although there has been some discussion of what ratings of hunger measure [61], the dissociation between effects of opioid antagonism and the use of preloads on rated hunger at the start of a meal clearly indicates that different mechanisms underlie these two manipulations of short-term eating. Thus, there is no evidence that opioid antagonists reduce motivation to eat at the onset of a meal. Animal studies are also consistent with this conclusion. For example, rats show the same

motivation to start feeding after naloxone and placebo [62], but eat less and more slowly after naloxone once feeding starts [63].

5. Opioids, food pleasantness and orosensory reward

Most models of short-term intake control consider meals as the consequence of two interacting feedback processes: a positive feedback process associated with stimulation of appetite through the sensory properties of the ingested food, and a negative feedback process associated with the stimulation of the multiple systems associated with satiation and satiety [51]. Opioids could potentially play a role in either or both of these processes. One way of testing these possibilities is to examine how sensitive the effects of opioid antagonism are to the sensory properties of the foods being eaten. Nine of the studies of the effects of opioid receptor antagonists on aspects of appetite in normal human subjects have included measures of rated pleasantness of either sucrose solutions [25,26,64], sweetened milk solutions [23] or actual foods [29,30,32]. All but one of these studies [28] reported significantly lower rated pleasantness following administration of an opioid antagonist than after placebo (Table 1). Hence, there appears to be a role for opioid receptors in some part of the neural circuitry underlying the expression of hedonic responses to food.

The effects of opioid antagonists on food pleasantness could, potentially, be secondary to changes in sensory perception induced by opioid antagonism. However, this is unlikely, since changes in hedonic evaluations have not been matched by changes in sensory evaluations [23,27,29, 32,64]. For example, neither the perceived saltiness of soups varying in salt content, nor sweetness of a soft drink varying in sucrose content, were altered by naltrexone, but in both cases perceived pleasantness was reduced [27]. Similarly, the pleasantness, but not rated sweetness or creaminess, of sweetened milk solutions was reduced by naloxone [23]. This lack of effect of opioid blockade on supra-threshold taste perception for simple stimuli is supported by a lack of change in the rated blandness, sweetness and saltiness ratings for food stimuli following naltrexone [29] and nalmefene [32]. Detailed evaluation of taste detection or recognition thresholds for four of the basic tastes (sweet, sour, bitter and salty) were also unaltered by naltrexone, but again a small, but significant reduction in the rated pleasantness of these stimuli was found [64]. Likewise, naltrexone failed to alter the rated sweetness or bitterness of sucrose and quinine solutions [65]. Most recently, the effects of naltrexone on both gustatory and olfactory evaluations of super-threshold stimuli have been examined [66], and no change in taste of classic tastants (sucrose, quinine, citric acid, sodium chloride) or the smell of orange extract or a cola extract were found. The same study also examined the effects of naltrexone on the taste and smell of ethanol, and again no differences in perception between

placebo and naltrexone were found. Thus, no study has been able to detect any change in gustatory perception either at threshold or supra-threshold levels following opioid blockade. This seems surprising, given the high concentration of opioid receptors in the neural circuitry associated with olfactory perception. Indeed, the concentration of δ receptors in the rat olfactory bulb is higher than anywhere else in the central nervous system [67], and both μ and κ receptors are located in the olfactory system. Although the limited study of the effects of opioid antagonists on olfactory perception have found no evidence for a role of opioids, recent work using an opioid agonist suggests that opioids may be involved in olfactory perception. Infusion of the μ-opioid receptor agonist remifantanil increased detection thresholds for a variety of odours, while having no significant effect on odour discrimination or recognition [68], and since μ-receptors are found in the olfactory system this is consistent with opioid involvement in olfactory perception. Administration of remifantanil did, however, cause other opioid effects (sleepiness, vomiting) and a non-specific account of these effects could not be discounted. As yet, no drugs which are specific at the κ or δ opioid receptor have been tested in humans in this context. Hence, while there is good evidence that opioids play a role in the development of olfaction in animals [69,70], the evidence in humans is as yet unclear, although the neuroanatomical distribution of opioid receptors alone suggests that opioids do play a role in olfaction that warrants further research.

In summary, opioid antagonists cause a small, but consistent reduction in the rated pleasantness of foods, tastants and odours, while having little detectable effect on gustatory or olfactory perception. While a role of opioids in olfactory perception cannot be dismissed from the evidence to date, any such effects appear unlikely to explain the reported effects of opioids and opioid antagonists on eating.

6. Opioids, alliesthesia and sensory-specific satiety

Since opioid antagonists do not suppress rated appetite (hunger) but do reduce food intake and flavour pleasantness, it has been suggested that opioids may normally be involved in the stimulation of appetite through palatability [4]. This idea is consistent with the animal literature, which has highlighted the role of palatability in determining the magnitude of the effects of opioid antagonism on food intake [71].

Although ratings of food pleasantness could be taken as a measure of orosensory reward in humans, some caution is needed in the interpretation of pleasantness ratings. One influential, and widely cited, finding is that the rated pleasantness of sweet tastes decreases when the consumer is sated relative to when they are hungry (negative gustatory alliesthesia: [72]). Similarly, pleasantness ratings for complex foods are consistently lower at the end of a meal than at the start [59,73,74], although this does depend to some extent on the specific phrasing of the rating [75,76].

The observation that this decrease in pleasantness is less evident with uneaten than with eaten items led to the concept of sensory-specific satiety [77]. Could the effects of opioid antagonists on rated taste and food pleasantness be interpreted as some action on the appetite control systems associated with the phenomena of alliesthesia and/or sensory-specific satiety? In both cases, this seems unlikely. If the reduction in pleasantness following opioid antagonism reflected enhanced alliesthesia, then the effects of antagonism should be found when sated, but not hungry. Although potentiated alliesthesia was reported in one study [78], all other studies found reductions in rated pleasantness in the absence of any change in rated hunger or prior to any food intake [25,29,32]. Likewise, sensory-specific satiety implies that reductions in pleasantness should be seen differentially for consumed items, but reductions in rated pleasantness associated with opioid antagonism are evident in the absence of food consumption [23,25,27,29,32,64]. These data imply that the changes in rated pleasantness associated with opioid antagonism reflect a more general modulation of the neural systems underlying hedonic evaluation of foods rather than alteration of the specific appetite controls associated with alliesthesia or sensory-specific satiety.

7. The opioid-palatability hypothesis

The evidence discussed to date suggests that opioid peptides play a specific role in some aspect of the neural system underlying the expression of palatability, the opioid-palatability hypothesis. In order to evaluate fully this idea in humans, an alternative measure of orosensory reward is needed in order to get around the potential problems of interpreting hedonic rating data. One approach has been to examine in detail changes both in eating rate and the pattern of changes in subjective appetite during meals following manipulations of palatability alone [79]. Increasing the rated palatability of a food increased intake and also resulted in increases in rated hunger during the early stages of the test meal [80]. One approach to examining these complex data was to model the changes in rated appetite as a function of cumulative intake within the meal. The resulting relationship was best described by a quadratic function with the three components of the function appearing to map onto separate aspects of short-term feeding motivation. Flavour modification only altered the linear component of this function [79], suggesting that this component is sensitive to palatability manipulations. This approach to studying human eating is similar to that developed in animal research [81], and makes a useful model for dissociation of different motivational influences on eating [79]. The opioid-palatability hypothesis was tested using this model by examining the effects of the opioid antagonist naltrexone on intake, eating rate and changes in subjective appetite within a test meal on a baseline (pre-test) day, and after both a

placebo and a naltrexone treatment, administered double-blind [30]. The opioid-palatability hypothesis predicted that opioid blockade should reduce intake and attenuate the short-term stimulation of appetite through palatability indicated by the linear component of the best-fit quadratic function. The results of this study were consistent with this prediction since subjects not only ate less after naltrexone (Fig. 1a), they also no longer showed any increase in appetite during the early stages of eating (Fig. 1b). Additionally, the study tested two different foods (pasta with a less palatable cheese sauce and more palatable tomato sauce: Fig. 1), and the effects of naltrexone were more evident with the more palatable version. These data clearly suggest that opioid release underlies the ability of palatability to stimulate appetite in humans.

8. Does eating palatable foods release opioid peptides?

The opioid-palatability hypothesis suggests that the sensory experience of palatable foods can, under certain conditions, lead to release of opioid peptides which in turn stimulate intake. Consequently, researchers have tested whether higher opioid levels are detectable after palatable foods have been eaten. In animals, ingestion of palatable food activated β-endorphin in the hypothalamus [82], and dietary-induced obesity in rats was associated with increased β-endorphin immunoreactivity in the pituitary [83], while feeding stimulated by altered food palatability increased hypothalamic dynorphin and associated mRNA levels [84]. More recently, cerebrospinal fluid (CSF) β-endorphin levels were increased after free drinking of sweetened solutions (sucrose and saccharin) and water, but not quinine and salt, in rats [85]. Thus, evidence from animal studies is consistent with palatability-induced opioid release both for endogenous μ- and κ- agonists. Most studies with humans have concentrated on one opioid peptide, β-endorphin, primarily because it is present in plasma, and is therefore relatively easy to assay. The earliest relevant study reported that β-endorphin levels were three times higher in obese subjects than normals [86], and this was confirmed in a follow-up study [87]. These results could be interpreted as heightened β-endorphin levels as a consequence of over-consumption of palatable food. However, other explanations are possible. For example, β-endorphin levels are known to be increased by stress as part of the general stress response, and although in these studies having a diagnosed affective disorder did not explain the observed results [87], it remains possible that the obese group were more stressed than the controls. A different, if somewhat unconventional, approach was taken by Melchior et al. [88]. They allowed subjects to select their favourite food from a delicatessen. The food was then eaten on two occasions, once when heated to the correct serving temperature, and once when frozen (ostensibly to remove sensory cues). β-Endorphin levels increased in the latter

A) Total intake

B) Hunger ratings
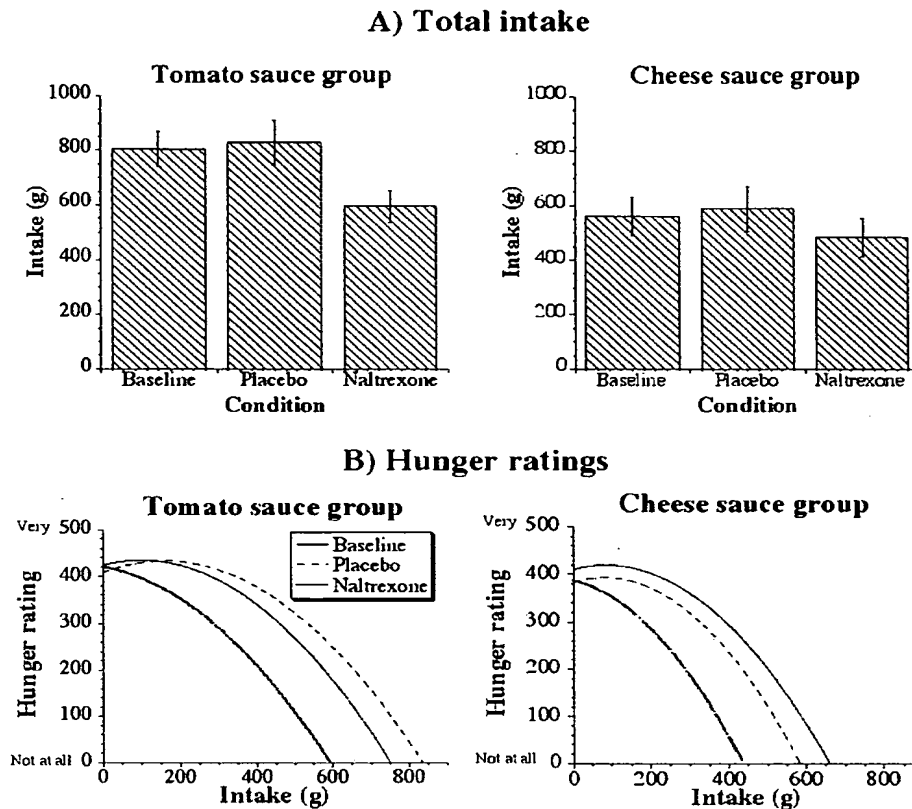
Fig. 1. The effects of naltrexone on food intake and rated hunger for subjects eating a pasta lunch with a cheese or tomato sauce. See Ref. [30] for details.

condition, contrary to the opioid-palatability hypothesis. However, in this instance the β-endorphin response could be explained by the stressful experience of eating a preferred savoury food in a frozen state, emphasising more the limitation of using peripheral measures of opioid activity. The same group also failed to find any change in β-endorphin in response to consuming a palatable food in one study [89], but reported β-endorphin release stimulated by ingestion of a palatable food in a second study [90]. The successful study assayed β-endorphin levels for 3 h post-drink in 10 normal-weight subjects consuming two chocolate-flavoured drinks (one sweetened with aspartame and one with sucrose) or a control condition. β-endorphin levels were highest after the drink sweetened with aspartame, intermediate after the sucrose-sweetened drink and lowest in the control condition. If the aspartame-flavoured drink was more palatable than the sucrose drink (which was not reported), this could provide limited evidence for peripheral opioid release stimulated by palatability. Taken together, studies of the effects of palatable food consumption on plasma β-endorphin levels provide, at best, weak evidence that consumption of a palatable food elevates peripheral opioid levels. Indirect

evidence that consumption of palatable foods leads to opioid release came from a case report of an obese woman administered naltrexone as part of a weight control programme [91]. In this case, naltrexone induced symptoms characteristic of opiate withdrawal in the absence of any evidence that she had been abusing opiate drugs. The authors concluded that opioid over-activity was central to her obesity, which is consistent with the notion that excessive consumption of palatable foods could lead to disturbances in opioid control. However, this appears to be an isolated case, since no similar reports have been made despite a large literature on the effects of opioid antagonists on eating and weight control in obese patients [92–96].

In summary, studies of opioid release during eating provide little support for the opioid-palatability hypothesis. However, the concentration on peripheral opioid levels is a major shortcoming of most studies in this area. β-endorphin is released as part of the general stress response, and plasma β-endorphin may better reflect the status of the stress system than central opioid release stimulated by eating. What is needed is a direct measure of central opioid release during eating, ideally in a form that allows direct evaluation of the involvement

of specific opioid receptor subtypes. Advances in neural imaging techniques have made such studies more feasible, and future research should now focus on this issue.

9. Are effects of opioid antagonists macronutrient-specific in humans?

One of the key findings used to support the opioid-palatability hypothesis is that opioid antagonists selectively reduced the relative intake of foods rated as more pleasant on a hedonic scale [31,32]. It is also possible to classify these foods in terms of macronutrient content rather than pleasantness, and since high-fat high-protein foods tend to be rated as more palatable, the effect on macronutrient intake is to reduce fat and protein intake, but spare the intake of non-sweet carbohydrate [31,32]. On the contrary, when a wide range of snack-type foods were tested, those which were rated as palatable, but which were largely non-sweet carbohydrate were almost unaffected by opioid antagonism with naloxone [23], whereas intake of palatable foods which were rich in fat and sugar were greatly reduced. This implies that opioids may have a macronutrient-specific role in the control of fat and sugar intake either in addition to a role in palatability, or even as an alternative to the putative role of opioids in palatability [23]. However, subsequent studies suggested that under certain circumstances intake of sweet foods was spared even when rated palatability of those foods was reduced [29]. The likely explanation for these conflicting findings is in the design of the eating tests, where habitual eating patterns, such as switching to a dessert partway through a meal, may confound drug-induced changes in appetite. Although a role for opioids in the control of fat and protein intake cannot be dismissed entirely, current data are more consistent with an effect on palatability than with any macronutrient specific explanation.

10. Opioids and disordered eating

The emergence of evidence that opioids are involved in appetite control led to a variety of investigations into the potential role of the opioid system in obesity and the major eating disorders anorexia nervosa, bulimia nervosa and most recently binge eating disorder. The earliest studies examined the possibility that opioid antagonists may have therapeutic value in the treatment of obesity, but although initial results were promising [92,97], further evaluation suggested that long-term treatment with opioid antagonists was ineffective as a weight control measure [93,94,98]. Since few of these clinical studies included any detailed analysis of eating behaviour, and the outcome of these studies has been reviewed in detail elsewhere [18,99], we do not re-examine these data here. Instead the present review

concentrates on those studies of obesity and disordered eating which include some measures of appetite control, since the outcome of these studies allow clearer evaluation of the role of opioids in eating in general. There have also been a few evaluations of the effects of opiate antagonists on food intake in sufferers from Prader–Willi syndrome, a congenital disorder associated with overeating and obesity [100–102]. Since these studies have been adequately reviewed elsewhere [18], and the outcome does not elucidate the role of opioids in eating controls, these studies are not reviewed further here.

11. Opioids and appetite control in obesity

Six studies have examined the effects of opiate antagonists on food intake and other measures of appetite in obese humans (Table 2). Five of the six studies testing the general obese population reported an average decrease in short-term food intake of 33% (averaged across drugs and doses, and adjusted for sample size), which is marginally higher than the reduction seen across studies in normals (21%, Table 1). Whether this difference reflects the larger doses of antagonist used in many of these studies, or is a consequence of opioids being more important in control of food intake in obesity, cannot be determined from these data. In contrast to the data from normals, where hunger at the onset of feeding was never modified by opioid antagonism (Table 1), two of the three studies to include ratings of appetite following administration of opiate antagonists to obese subjects reported suppressed rated hunger at the onset of eating. This could be interpreted as a difference in appetite control mechanisms between obese and normals, but alternatively may be an indirect consequence of the differences in methodology between studies in normals and obese patients. For example, the study testing naltrexone in obese patients [103] assessed eating over a 4 week period, with naltrexone administered for 7 days and the naltrexone dose increasing from either 25 or 50 mg to 100 or 200 mg. No effect of naltrexone on rated hunger or intake was seen on day one, but both hunger and intake were reduced on subsequent days, when the naltrexone dose was higher than that used in the studies with normals. Thus this study differed from acute studies in normals both by employing a chronic treatment regime and using higher doses of naltrexone, and both these factors could explain why hunger was affected here, but not in normals. Of the two studies using naloxone, the study finding an effect on hunger [95] did not explicitly test whether there was a difference between placebo and naloxone conditions prior to the test meal, but instead reported an attenuated increase in hunger across the infusion period in the naloxone condition relative to placebo. Given that the study reporting no effect of rated hunger at the onset of a meal in obese subjects [23] used a larger sample size [9, 16], the evidence that naloxone reduces general appetite in

Table 2
Effects of opiate antagonists on food intake, rated hunger and food pleasantness in obese subjects

Reference	Subjects	Drug, route and dose	Effects on appetite		
			Food intake	Hunger at start of meal	Rated food pleasantness
[92]	7 men and 7 women	Naloxone IV, 5 mg bolus + 5 mg/h for 2 h	29% decrease	Not tested	Not tested
[97]	5 women and 1 man	Naloxone, IV 0.5 mg/kg	25% decrease	Not tested	Not tested
[96]	8 men	Naltrexone oral, 100, 200 and 300 mg	Non-significant decrease	Not tested	Not tested
[103]	18 men	Naltrexone oral, rising from 25 to 200 mg max over 7 days	Peak 45% decrease	Decreased	Decreased
[95]	8 women and 1 man	0.5–2.0 mg/kg naloxone IV	38% decrease	Decreased	Not tested
[23]	16 women	Naloxone, IV 6 mg + 0.1 mg/kg 2.5 h	22% decrease	No effect	Decreased
		Butorphanol, IV 1 μg/kg	9% decrease (NS)	No effect	No effect

obese subjects is far from convincing. It is also notable that most of the studies using obese subjects reported side effects of drug-administration, most notably nausea, even with naloxone [95]. It is possible that the enhanced anorectic effect and reduced hunger in some of these studies may therefore be attributed to non-specific drug effects in the obese population.

Obesity is now recognised as a consequence of a positive energy balance associated with excessive energy intake and augmented by low energy expenditure. One possible explanation is that obesity represents an excessive stimulation of appetite by palatability, and the evidence for opioid involvement in palatability discussed earlier in this review is consistent with this idea. Also, excessive levels of plasma β-endorphin have been reported in obese patients [87], and the only reported case of opiate-withdrawal syndrome after administration of naltrexone in the absence of opiate-abuse was in an obese woman [91]. It is also the case that as palatable food has become more readily available, the incidence of obesity has increased. Thus the idea that hedonically-driven over-eating may be a significant contributor to obesity remains a plausible idea.

12. Opioids and appetite disturbance in anorexia nervosa

The idea that disturbance to the opioid systems involved in appetite control might contribute to anorexia nervosa was first developed in the late 1970s [104]. These early observations of disordered opioid function in anorexic patients, coupled with the observation that anorexia becomes increasingly intractable, resulted in a specific theory of the neurochemical basis of the chronic anorexic state [105]. In brief, this model suggests three separate roles for opioids in disordered eating. First, the model suggests that initial dieting releases opioids which induce positive mood, a claim which is consistent with the

opioid-palatability hypothesis. Secondly, a separate action of opioids increase the desire to eat to correct the self-induced starvation. Finally, opioids are also heavily implicated in the adaptation to self-starvation, with a variety of effects aimed at reducing energy expenditure. The role of opioids in disordered eating will depend crucially on the relative activity of these three different processes. Persistent anorexic behaviour could be seen as an addiction to the starvation process, whereby the first and third effects of opioids predominate, whereas bulimia is seen in this model as an addiction to the starvation-induced desire to eat. Evidence for opioid dysfunction in anorexia has been found. For example, CSF opioid activity was found to be high in anorexic patients, and this abnormality normalised when weight was restored [106]. However, plasma β-endorphin levels have been found to be higher in anorexic patients in most studies [107,108], but lower in other studies [109]. Thus, opioid activity appears abnormal, and these differences could be explained by an auto-addiction model, but not all of the data on opioid disturbance in anorexia is consistent with this model: Krahn et al. [110] reported the case history of a young woman with combined problems of heroin abuse and anorexia nervosa. According to the auto-addiction model, stabilising this woman's opiate habit by long-term naltrexone therapy should have also led to improvement in her anorexic behaviour. However, the reverse was true: naltrexone blocked heroin addiction (as it should) but exaggerated her anorexia nervosa. When she came off naltrexone, her eating disorder improved, but she lapsed back to her heroin habit. Thus, her case casts doubt over the auto-addiction model. However, studies using naltrexone therapy have reported positive effects on anorexic patients [111,112]. It remains possible that opioid dysfunction may underlie some aspects of anorexic behaviour, although whether this reflects the complex role for opioids suggested by the auto-addiction model is unclear.

Although most interest on the role of opioids in anorexia nervosa have focussed on the effects of chronic disturbance

to the opioid system, an alternative perspective is that anorexia nervosa arises as a pathological consequence of a primitive opioid-mediated mechanism which evolved to help cope with unforeseen short-term food shortage, including mediation of the short-term energy balance adjustments discussed earlier along with masking or alleviating negative mood associated with food deprivation [99]. This suggestion can be tied into the potential role of opioids in stress-induced eating [113], but the complexity and inconsistency in the literature on disruption to the opioid system in anorexia makes it difficult to fully evaluate this model.

13. Opioids and binge-eating behaviour

While the case for a role for opioids in anorexia nervosa remains unclear, the case for a role in bulimic behaviour is more compelling. The parallels between binge-eating behaviour and substance abuse were highlighted by Hardy and Waller [114], who emphasised that aspects of binge eating could meet the DSMIII diagnostic criteria for substance abuse, and who discussed the possibility that opioid dysfunction could underlie addictive binge eating. There is a variety of evidence in support of this hypothesis beyond the generalised data on opioid function in hedonic aspects of eating already discussed in this review. First, a number of studies have reported abnormal levels of β-endorphin in the plasma or CSF of binge eaters. Most reports suggested that β-endorphin levels in CSF are lower than normal in bulimic patients [115], while plasma levels have also been lower in some studies [116], although higher in others [117]. The difference in plasma levels between studies could be explained either by differences between vomiting and non-vomiting patients, with higher levels of β-endorphin in the former as a response to the stress of vomiting, or alternatively it could reflect different sampling points, perhaps with lower β-endorphin levels prior to a binge and higher after. Either way, most published studies do show abnormal β-endorphin levels, in line with the idea of disrupted functioning. A second line of evidence lending support to an opioid-binge hypothesis is that opioid blockade can reduce the frequency and severity of binge eating, although not all studies find these effects. Early studies using open-label designs were promising, with reduced binge size and frequency following naltrexone [118–120]. Intravenous naloxone infused double-blind also has a positive effect [121], with a 23% reduction in energy intake, although notably no effect on the time spent bingeing. More recently, a double-blind placebo-controlled study with 100 mg naltrexone extended these findings [122], with 18/19 bulimic patients showing improvements in behaviour, including reduced binges and purges, and a reduced ratio of binge to normal eating. Similarly, 100 mg naltrexone reduced binge duration in bulimic patients, and tended to do so also in obese binge-eaters [123]. However,

other double-blind studies have failed to find effects of naltrexone on long-term food intake in bulimic patients [124]. Given the role of opioids in perceived food pleasantness, the effects of opioid antagonists on binge eating could be explained by over-responsiveness of binge eaters to hedonic aspects of eating. Thus, if satiety cues are ignored then meal-size may be determined primarily by hedonic stimulation of appetite, and (as discussed earlier) current evidence clearly implicates opioid peptides in this hedonic stimulation. Explicit evaluation of this idea would require analysis of orosensory reward processes during a binge meal. Although there is a growing literature examining binge eating in the laboratory [125,126], to date, studies of the effects of opioid antagonists on binge eating have lacked the behavioural sensitivity needed to evaluate properly the idea that binge-eating may be driven, at least partially, by opioid-mediated hedonic stimulation of appetite.

14. Opioids and the control of drinking: water intoxication as water addiction

As well as altering feeding, opioid antagonists alter normal drinking behaviour in animals [2]. No equivalent reports have been made in humans; however, there is some evidence for opioid involvement in compulsive over-drinking of water, otherwise known as psychogenic polydipsia. This condition is most common in schizophrenic and other psychiatric patients, and involves excessive water intake to a point where the patient is at risk of self-harm through over-hydration [127]. Starting with a case study, Nishikawa and colleagues [128] have provided consistent evidence of a therapeutic benefit following naloxone administration both in open trials [129] and a double-blind trial [130]. Although only some patients showed decreased drinking following naloxone, these effects were dramatic in certain instances. There is no clear explanation why only some patients responded to naloxone, but it is possible that polydipsia has multiple causes, a suggestion supported by more recent reports that the atypical antipsychotic drugs resperidone and olanzapine and angiotensin receptor blocker irbesartan also have therapeutic value [131]. The ability of naloxone to reduce a behaviour which might be interpreted as water addiction fits well with the idea that opioids play an important role in addictive elements of ingestive behaviour.

15. Opioids and alcohol abuse

As with the other aspects of ingestive behaviour reviewed here, there is increasing evidence that opioid peptides play some role in controlling alcohol intake. Indeed, initial reports that naltrexone taken daily after detoxification in alcoholics can reduce the frequency of

subsequent drinking and relapse to heavy drinking [132, 133] led both to the approval for the general use of naltrexone in the treatment of alcoholism and subsequently a large and growing clinical literature which, in general, has confirmed the original findings [133–138], and identified the conditions under which opiate antagonism has the greatest clinical efficacy. A full review of the clinical literature is beyond the scope of the present review, and has been the subject of several authoritative reviews [139–143]. However, the basis of the observed effects of naltrexone and nalmefene is pertinent since it is possible that the mechanism by which opiate antagonists help alcoholics avoid relapse to heavy drinking is related to the mechanisms underlying opioid involvement in the control of eating and drinking.

As yet, no consensus on the basis of the effects of opioid antagonism in alcoholics has emerged. If this effect were the basis of a shared mechanism with that seen in eating, then the prediction would be that the clinical efficacy of opioid antagonists in recovering alcoholics could be based on reduction in the hedonic consequences of alcohol ingestion. Alcohol would be predicted to taste less pleasant, and the hedonic experience of drinking ameliorated. Although the literature in this area is still limited, there are reports in the literature which are consistent with these predictions. First, in clinical studies alcoholics who sampled alcohol reported less hedonic effects than they expected [144]. However, laboratory tests of the effects of opioid antagonists on a variety of measures of the behavioural effects of alcohol have had mixed outcomes. The results of a number of these studies are consistent with a role of opioids in hedonic aspects of alcohol consumption. For example, chronic dosing with naltrexone significantly decreased ratings of liking for the effects of alcohol, decreased best-effects of alcohol and reduced desire to drink [145]. Naltrexone-treated subjects also consumed less alcohol and drank more slowly [146]. In another study, naltrexone reduced intake of both an alcoholic and non-alcoholic control drink, while having no effect on the subjective experience of alcohol [147]. In that study, no measure of drink pleasantness was reported, but assuming that both the alcoholic and placebo drink were palatable, the effects on intake would be consistent with the literature on eating discussed earlier. However, the result of other studies goes against a hedonic explanation. For example, no effect of naltrexone on amount consumed, liking for the effects of alcohol or time to consume a drink were reported [148], but instead latency to start drinking was increased. These data are contrary to the outcome of studies on eating, where motivation to eat at the start is unaffected, but duration and liking are reduced. Several studies found no effect of naltrexone on either positive mood induced by alcohol or liking for the post-ingestive effects of alcohol [149–151]. Thus, the evidence that opioids are involved in the perceived post-ingestive effects of alcohol in humans is very limited, but there is some evidence that opioid

antagonism does modify the experience of drinking and amount consumed.

A second measure which has been used in several laboratory studies of the effects of opioid antagonism on alcohol drinking is that of craving for alcohol. Recent models of drug craving are based around the idea that cravings are conditioned responses to drug-associated cues [152]. Naltrexone has been reported to reduce measures of craving or urge to drink for alcohol in several studies [134, 145,146], and was more effective in patients who had high baseline craving for alcohol [153]. Another recent experimental study suggests that naltrexone reduced these cue-elicited cravings for alcohol [154]. However, since reduced urge-to-drink could be due to reduced positive effects of alcohol, enhanced aversive effects of alcohol, or aversive effects of naltrexone alone [145], the overall picture on the effects of naltrexone on craving for alcohol remains unclear.

An alternative explanation for the efficacy of naltrexone in the treatment of alcohol dependence is that naltrexone has non-specific effects which in turn make alcohol less attractive. Many of the clinical reports into the effects of naltrexone on alcohol dependence have reported side effects of naltrexone, with drug-induced nausea particularly prevalent [35]. However, these side effects appear to have a stronger effect on study compliance than on drug efficacy in treatment of alcohol. For example, in one study only those patients who were highly compliant showed an advantage of naltrexone over placebo [155], and high levels of adverse reactions to naltrexone led to poor medication compliance, and no advantage of naltrexone over placebo, in a recent clinical trial [156]. Thus, side effects may lead to poor compliance with medication, although the finding that patients who remain compliant do, in most studies, shows a benefit of naltrexone medication questions whether these non-specific effects can explain the therapeutic benefit. Non-specific effects could explain some of the variability in laboratory-based studies. For example, naltrexone-induced nausea and fatigue may explain the enhanced latency to drink in a laboratory test [148]. Other research suggests that naltrexone enhances the aversive qualities of alcohol, with the suggestion that alcohol may itself induce nausea under the influence of alcohol [145]. Thus, the effects of naltrexone on alcohol consumption may combine two different effects, one relating to the role of opioids in hedonic components of alcohol consumption, which may be blunted by naltrexone, and another whereby adverse effects of alcohol (nausea) are enhanced by naltrexone. The overall consequence of these combined effects is to make the drinking experience less pleasant, so enhancing the ability of recovering alcoholics to abstain from drinking. Further research is needed to validate this suggestion.

In contrast to the complex picture emerging from the limited human literature on the nature of opioid involvement in the control of alcohol intake, there is strong evidence for opioid involvement in the control of alcohol intake in animal models [3,157]. Naloxone reduces alcohol

intake in rats in a wide range of alcohol drinking models [158,159–162]. A point of controversy is whether this reduction in intake is specific to the alcoholic drink or is also seen with a non-alcoholic control drink, with some studies reporting specificity [163] and others non-specific reduction in drinking [158,164]. One possibility is, in line with the literature on opioid involvement in eating and intake of non-alcoholic solutions is that these results depend on the relative palatability of the two test drinks, with selective effects seen on alcoholic drinks where these have higher palatability than the control drink. Where the drinks tested alongside alcohol were sucrose and saccharin, non-specific reductions were reported [158], and it is long established that opioid antagonism greatly reduces intake of sweet solutions by rats [12,13,165,166]. Thus, the effects of opioid antagonists on intake of alcohol in animals show similarities to effects on feeding and drinking in general. More direct evidence for an effect of naltrexone on the palatability of an alcoholic drink in rats came from studies using the taste-reactivity test [167], where naltrexone increased aversive responding to alcohol [168]. Further evidence that opioids play an essential role in the control of normal alcohol intake was the finding that mice lacking the μ-opioid receptor failed to self-administer alcohol [169], while pre-treatment of the high-alcohol drinking (AA) strain of rats with naltrexone caused upregulation of brain opioid receptors [170]. Overall these data provide strong evidence for opioid involvement in the control of alcohol intake in rats most of which is consistent with the hypothesis that opioid reward mechanisms are an essential element of the control of alcohol intake [3].

At present there appears a disparity between the animal and human literature on the role of opioids in the control of alcohol intake, with the balance of evidence suggesting that opioid reward mechanisms play an essential role in alcohol-drinking in animals, but a much less clear position in humans.

16. Future research directions

Re-evaluation of the literature on the role of opioids in human eating in light of the increased clinical use of naltrexone in the treatment of alcohol dependence has identified some interesting similarities and differences between these two sets of findings. It also clarifies where further research is needed. Both literatures are complex, and in both cases a simple single mechanism for the role of opioids in the control of ingestive behaviour is clearly unlikely. This is not surprising given that opioid receptors are widely distributed in the central nervous system, and it is clearly possible (and indeed likely) that more than one brain circuit involving opioids will be involved in both normal eating and alcohol consumption. One circuit which may be shared across eating and alcohol intake is a brain opioid reward circuit which is involved in hedonic aspects of both

eating and drinking. However, few of the studies on alcohol intake include any direct measure of how opioid antagonism alters the immediate experience of the actual drink, instead measuring liking for the experience of alcohol. In contrast, animal studies explicitly suggest that opioid antagonism is only effective if alcohol is experienced during opioid blockade [157]. An important element of future studies on effects of opioid antagonism in relation to alcohol dependence is to examine in more detail how opiate antagonists modify the perceived characteristics of alcoholic drinks. Studies have shown no change in some aspects of sensory perception [66], but no study that we are aware of has examined how opiate antagonists alter the perception of drink pleasantness.

An area which has been neglected, but which warrants further investigation, is the interaction between eating and alcohol-drinking. This encompasses two areas of relevance to this review. First is the issue of how alcohol consumption itself modifies appetite, and whether the opioid mechanisms discussed here are involved. Secondly, if opioid antagonists alter appetite, does this itself raise issues about the use of these drugs therapeutically in treatment of alcohol dependence. Laboratory-based studies have repeatedly found a failure for participants to reduce food intake in response to the energy in alcohol at a test meal taken with or soon after alcohol intake [171–174]. More recent studies suggest that, in fact, alcohol may go further and actually have a short-term stimulatory effect on appetite [175–178]. Although some of this short-term increase may be explained by psychological consequences of the relaxing effects of alcohol [179], other studies suggest that at least some of this stimulatory effect of alcohol is due to a pharmacological effect of alcohol [175,177]. The mechanism underlying this short-term stimulation of appetite remains unclear, but since alcohol ingestion leads to central opioid release in animals [84,85], and this review has highlighted the role of opioids in enhancing short-term food intake, a role of opioids in the stimulatory effect of alcohol on appetite is clearly possible, and warrants further investigation. The second issue, the implications of opioid involvement in the control of eating for the current therapeutic use of opiate antagonists in treatment of alcohol dependence, is even less clear. The direct implications of studies of the effects of naltrexone on food intake and appetite in people is that in the short-term alcohol-dependent patients treated with opiate antagonists should experience reduced short-term food intake and should find food less enjoyable. However, since the studies on appetite has been conducted in normals, whether this applies to recovering alcoholics is untested, and even if short-term appetite was affected whether this effect was maintained during chronic treatment with opiate antagonist is unknown. The outcome from studies with obese patients (reviewed earlier), however, suggest that long-term appetite will be unaffected in alcohol-dependent patients, since opiate antagonists failed to reduce body-weight despite having short-term effects on appetite.

Another area warranting new research is whether genetic variability in opioid receptors may help explain variability in the tendency for individuals to become obese, binge-eat or develop alcohol dependence. Specific polymorphisms of the μ receptor have been reported in people [180,181]. Whether these polymorphisms map onto specific patterns of food intake, body-size, food preferences or alcohol use remain largely untested, although the possibility of linkage between opioid polymorphisms and alcohol dependence is being explored [182], although initial results argue against an association between identified polymorphisms and alcohol dependence [181]. Some interactions between feeding and alcohol dependence are worth noting here. For example, the idea that excessive sweet preference may be associated with obesity has long been discussed, and although this idea is not well supported in general [183, 184], desire for sweet taste may be a risk factor for the development of obesity in particular populations [185]. Recent data also suggest that sweet preference is higher in alcohol-dependent subjects [186], and that enhanced sweet-taste preference may be a risk factor for the development of alcohol dependence [187]. As this review has noted, opiate antagonists reduce liking for sweet tastes in humans and animals. This implies that opioids are important in the expression of sweet taste preference, and it then follows that individuals with enhanced sweet taste preference may have some genetic abnormality to their opioid system, and future research might explore this potential interaction. Indeed, the increased sophistication of modern genetic techniques should allow substantial progress to be made in the evaluation of the role of genetic polymorphisms of the opioid systems as explanations for variability in the expression of ingestive behaviours.

Finally, a continued area for concern is the extent to which the outcome of studies with opiate antagonists can be interpreted as evidence for a direct role of opioids or are a consequence of the side effects associated with administration of these drugs. This issue has been explored at length both for studies on eating and treatment of alcoholism earlier in this review. Although we feel the balance of evidence suggests that behavioural effects of opioid antagonists have been demonstrated independently of side effects, future research should take care to include measures of non-specific drug effects and partial these out in analysis of behavioural variables to ensure that these data are interpreted correctly.

17. Summary and conclusions

This review has examined evidence for a role of opioid peptides in the control of human ingestive behaviour, bringing together evidence from a wide range of studies. We have identified areas where data are inconsistent or lacking,

and where future research would be valuable. Overall, it is clear that any attempt to explain changes in food, water and alcohol consumption in response to pharmacological manipulation of opioid receptors as the result of a single action of opioid peptides is problematic until some of the shortcomings in the present literature are addressed. However, it is also clear that early suggestions that opioid peptides may play a role in hedonic aspects of feeding and fluid consumption [4] are still broadly supported by the literature, and that it is likely that the role of opioids in modulation of alcohol intake may be at least partially explained by a similar effect. Further evaluation of this hedonic component of ingestive behaviour may be useful both in terms of advancing our theoretical understanding of the role of reward in behaviour, but also in terms of the development of therapies for obesity, binge-eating and alcoholism.

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Opioid modulation of immune responses: effects on phagocyte and lymphoid cell populations

Toby K. Eisenstein *, Mary E. Hilburger [1]

Department of Microbiology and Immunology, Temple University School of Medicine, 3400 N. Broad Street, Philadelpha, PA 19140, USA

Abstract

The literature describing effects of morphine on cells of the immune system points to the clear conclusion that morphine given in vivo suppresses a variety of immune responses that involve the major cell types in the immune system, including natural killer (NK) cells, T cells, B cells, macrophages and polymorphonuclear leukocytes (PMNs). Depression of NK cell activity has been reported in humans, monkeys and rodents. Similarly, responses of T cells are depressed by morphine, as assessed by inhibition of induction of delayed-type hypersensitivity reactions and cytotoxic T-cell activity, modulation of T-cell antigen expression, and depression of responses to T-cell mitogens. Effects on T cells have been reported in humans, monkeys and rodents. Effects of morphine on B-cell activity have mainly been tested in rodents using assays of antibody formation, which also require macrophages and T cells, preventing a conclusion as to the cell type being affected. Consistent effects on phagocytic cell function have been reported in rodents given morphine. In contrast, studies on immunomodulatory effects of morphine added to cells of the immune system in vitro have shown robust effects on some of these cell types, but not others. There is a rich literature demonstrating downregulation of phagocytic cell function by morphine, particularly for human peripheral blood mononuclear cells (PBMCs) and PMNs. Phagocytosis, chemotactic responses, interleukin production, and generation of activated oxygen intermediates and arachidonic acid products have all been reported to be inhibited. On the contrary, the literature does not support direct effects of morphine on NK cell function, is inconclusive concerning effects on B cells, and provides limited evidence for effects on T cells. The divergence between the in vivo and in vitro data suggests that effects on some cells in the immune system observed after in vivo morphine are probably not direct, but mediated. In aggregate, the literature supports the existence of an in vivo neural-immune circuit through which morphine acts to depress the function of all cells of the immune system. Further, there is strong evidence that morphine can directly depress the function of macrophages and PMNs, and modulate expression of one type of T-cell surface marker. There is, however, little evidence for direct effects of morphine on NK cells and B cells. A further complication emerges from reports of immunopotentiation of immune function in in vitro assays using endogenous opioids. The possibility of different receptors for endogenous and exogenous opioids or of interactions among the activated opioid receptors may account for these opposing effects. © 1998 Elsevier Science B.V.

Keywords: Opioids; Morphine; Macrophages; Immunomodulation; Phagocytes; Immune responses

1. Introduction

Recognition in 1979 that opioids have effects on immune cell function was a major discovery whose implications are far-reaching and are still in the infancy of their

exploration. Among the consequences of the existence of a neuroimmune circuit involving opioid pathways is the possibility of immunomodulation in users and abusers of heroin, in patients receiving morphine or other opioids for relief of pain, and in the more difficult to assess arena of alterations in levels of endogenous opioids resulting from pain, inflammation, or stress. The most clear-cut evidence for immunomodulation by opioids has been obtained using morphine in rodents. In almost all of the reported studies, morphine was found to be immunosuppressive. The import of these findings is that morphine or heroin use may

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* Corresponding author. Tel.: + 1 215 7073585; fax: + 1 215 7077920; e-mail: tke@astro.ocis.temple.edu

[1] Present address: Laboratory of Tumor Biology, National Cancer Institute, Bethesdy, MD, USA.

sensitize to infection, including to HIV, due to impairment of immune function.

This review will assess the evidence pointing to the conclusion that opioids are immunomodulatory, with emphasis on what is known about effects of these substances on specific cell types in the immune system. In the short time that research has been evolving in opioid-immune cell interactions, a major issue in the field has been the question of whether the effects of opioids are directly on the immune system or are indirect via neuronal signals or other neural mediators. This issue surfaced because of the difficulty in demonstrating the existence of opioid receptors on lymphoid cells or macrophages, in spite of evidence of effects of the drugs on biological function (Sibinga and Goldstein, 1988). Because evidence for the presence of opioid receptors is a crucial part of the thesis that immune cell function is directly altered by opioids, some of the papers in this area will be briefly discussed, although another review in the journal will address receptors in greater depth.

The paper usually credited with the first demonstration that opioids affect immune function is that of Wybran et al. (1979), published in the Journal of Immunology. In this original work, the investigators showed that if morphine, an alkaloid opiate, was added to human peripheral blood mononuclear cells (PBMC) and incubated for 1 h, the T cells showed reduced rosetting with sheep red blood cells, indicating modulation of a surface receptor (now known to be CD2). The paper went on to demonstrate that the effect of morphine could be blocked by preincubation with the opioid antagonist, naloxone, providing evidence that the effect had pharmacologic specificity with characteristics of a classical opioid receptor. Interestingly, these investigators also tested the effect of the endogenous opioid peptide, methionine–enkephalin (met–enkephalin), and found that it had the opposite effect from morphine; it augmented T-cell rosetting. In the nervous system, several types of opioid receptors are recognized. They are designated mu (μ), kappa (κ), and delta (δ). Opioid agonists which have high affinity for different receptor types in the nervous system have different biological effects. For example, activation of μ-opioid receptors in most species results in hyperthermia, while activation of κ receptors causes hypothermia (Adler et al., 1988). Morphine binds with greatest affinity to the μ receptor and with lesser affinity to δ and then κ receptors (Paterson et al., 1983). Met–enkephalin, however, binds almost equally to μ and δ receptors (Paterson et al., 1983). Thus, the Wybran paper provided evidence for the existence of opioid receptors on lymphoid cells, and for the possibility that in the immune system, as in the nervous system, opioids with action at different opioid receptor types can result in different, and even opposite, biologic effects. In light of the subsequent controversy over whether immune cells have opioid receptors and whether the effects of opioids on immune function are due to direct or indirect effects on cells of the immune system, it is important to point out that the experiments of Wybran showed direct effects of opioids added to lymphoid cells in culture.

2. Effects of opioids on phagocytic cells

Phagocytic cells, including macrophages and polymorphonuclear leukocytes (PMNs), are important because they engulf and kill microbes. Macrophages also present antigen to lymphoid cells and produce cytokines that modulate the immune response. Alteration of phagocyte function by opioids would be expected to alter host defense to infection, and alterations of macrophage function might result in modulation of immune responses.

Tubaro et al. (1983) reported that mice injected with morphine for 3 days had impaired capacity of both PMNs and elicited peritoneal macrophages to ingest the yeast *Candida albicans*. Further, rabbits given morphine showed lower cell counts upon alveolar lavage. The macrophages that were harvested had impaired phagocytosis and killing of the yeast. Naloxone, a morphine antagonist, was not effective in blocking the suppressive effects of morphine, very likely due to the timing of its administration. Rojavin et al. (1993), using unelicited peritoneal macrophages from mice implanted with a slow-release morphine pellet, confirmed the inhibitory effect of the opioid on phagocytosis of *Candida* and extended the study to show that simultaneous administration of naltrexone (similar to naloxone) by a slow-release pellet blocked the suppressive effect of morphine. Rojavin also showed that morphine added in vitro to unelicited mouse macrophages suppressed phagocytic function towards *Candida*. Other in vivo studies using morphine, reported by Pacifici et al. (1994), found alterations in phagocytic function of elicited murine PMNs for *Candida*. This group is one of the few to have examined cells harvested at early time points after morphine pellet implantation. They reported that 20 or 40 min after morphine administration, there was an increase in phagocytosis and killing of this organism, whereas cells harvested 24 h later were suppressed in these functions. A similar biphasic effect was observed in the ability of elicited macrophages to mediate cytotoxicity against tumor targets. Opioid antagonists were not used in these studies. An interesting observation was made by Roy et al. (1991b). They found that morphine pellets inhibited the capacity of bone marrow macrophage precursors to develop in vitro into viable colonies in response to macrophage colony stimulating factor, and the effect was inhibited by naltrexone. Also, in vitro addition of morphine or β-endorphin to precursor cells had similar activity, showing that the action of morphine was directly on the precursor cells.

There are a large number of papers reporting effects of morphine or other opioids added directly to phagocytic cells. Szabo et al. (1993) found that treatment of unelicited mouse macrophages with μ, κ, or δ receptor-selective agonists ([D-Ala^2,N-Me-Phe^4,Gly-ol^5]enkephalin

(DAMGO or DAGO), U50,488H, and [D-Pen², D-Pen⁵] enkephalin (DPDPE), respectively) inhibited ingestion of *Candida*, and that antagonists selective for each receptor blocked the inhibition of the corresponding agonist. Casellas et al. (1991) reported that phagocytosis of sheep erythrocytes by elicited mouse macrophages was inhibited by morphine and by leu- and met–enkephalin, and the effect was naloxone reversible. Human macrophage chemotaxis was found to be inhibited in Boyden chambers by a 5-min incubation with morphine, DAGO or DPDPE (Perez-Castrillon et al., 1992). All opioid effects were naloxone-inhibitable. In confirmation of these results, the laboratory of Stefano found that morphine inhibited chemotaxis of human PBMC (Stefano et al., 1993) and granulocytes (Makman et al., 1995). Morphine blocked the activity of several chemotactic agents including Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), Interleukin-8 (IL-8), and N-formyl-met-leu-phe (fMLP) (Makman et al., 1995) and naloxone antagonized the effect. These observations are in contrast to the report of Ruff et al. (1985), who failed to find an effect of morphine on chemotaxis of human monocytes but observed an increase in locomotion when cells were exposed to [D-ala²-D-Leu⁵] enkephalin (DADLE), β-endorphin or dynorphin (κ-selective) over a range of doses. The group of Chuang (Liu et al., 1992) found that PMNs of Rhesus monkeys had impaired chemotactic activity after treatment with morphine in vitro. In summary, in all but one of the reports in the literature, morphine was found to be suppressive of phagocytosis and chemotaxis of phagocytic cells of animals and humans.

Other properties of phagocytic cells have also been shown to be inhibited by morphine. Peterson et al. observed that human PBMCs exposed to morphine in vitro had reduced production of superoxide and peroxide in response to exposure to concanavalin A (Peterson et al., 1987a) or to phorbol myristate (Peterson et al., 1989). However, the opioid effect was found to be on lymphocytes in the preparations, which released a macrophage-suppressive substance, later identified as probably being Transforming Growth Factor-β (TGF-β) (Chao et al., 1992). In a study by Peterson et al. (1989) methadone-maintained patients had similar impairment of capacity to generate superoxide, and methadone was also suppressive when added to normal cells in vitro. Mazzone et al. (1990) reported that morphine blocked human granulocyte aggregation induced by lectins or fMLP, and also secretion of arachidonic acid products. However, only the cell aggregation effect was blocked by naloxone.

In PBMCs, morphine has been shown to increase TGF-β production by cells stimulated with lipopolysaccharide (LPS) or phytohemagglutinin (PHA) (Chao et al., 1992). However, morphine inhibited IFN-γ production by PBMC stimulated with Concanavalin A (Con A) or varicella zoster virus. In this case, the effect of the morphine appeared to be directly on the macrophages, and to be

mediated by macrophage reactive oxygen intermediates and prostaglandin E₂ (Peterson et al., 1987b). Morphine also blocked the release of bioactive tumor necrosis factor (TNF) from PBMC cultures treated with LPS or PHA (Chao et al., 1993). The synthetic κ-opioid agonist, U50, 488H has also been shown to inhibit LPS-driven IL-1 and TNF-α release from the murine macrophage cell line P388D1 (Belkowski et al., 1995a), and from primary murine peritoneal macrophages (Alicea et al., 1996). However, Interleukin-6 (IL-6) levels were unaffected in the primary cultures (Alicea et al., 1996). All of these effects on cytokines were shown to be opioid receptor-mediated using appropriate antagonists.

Interestingly, almost all of the effects of morphine and of nonpeptide κ-opioids on phagocytes are inhibitory. Some effects of opioid peptides are also inhibitory, for example, on phagocytosis of yeast (Szabo et al., 1993). In contrast, several studies reported that opioid peptides that bind at various opioid receptor types are stimulatory in a variety of assays. For example, Ruff et al. (1985) reported that DADLE, β-endorphin, and dynorphin (1–13) were stimulators of human mononuclear cell chemotaxis. This finding was confirmed and extended to show that DADLE, DAGO, DPDPE, D-Ala², Met⁵-enkephalinamide (DAMA), dynorphin A(1–17), D-Ala²-deltorphin 1 and met–enkephalin were all chemotactic for human granulocytes (Makman et al., 1995). Dynorphin A has also been reported to enhance tumoricidal activity of mouse macrophages (Foster and Moore, 1987; Hagi et al., 1994) and to stimulate superoxide production of human PMNs and peritoneal macrophages (Sharp et al., 1985). β-endorphin was also active in stimulating superoxide activity (Sharp et al., 1985).

Demonstration of opioid receptors on macrophages and granulocytes has provided a definitive pharmacological mechanism for the observed biological effects of morphine. The κ-opioid sites were described on P388D1 cells using classical binding approaches with radiolabelled ligands (Carr et al., 1991). A μ receptor has been cloned and sequenced and found to be identical to the brain opioid receptor (Sedqi et al., 1995). Human granulocytes have been shown to have a receptor for morphine that has been classified as the μ₃ subtype (Makman et al., 1995), and also to have δ₁ and δ₂ opioid-binding sites (Stefano et al., 1992). Based on binding studies, it has been proposed that the μ receptors on human granulocytes are different from peptide-binding sites on these cells (Makman et al., 1995). Existence of two types of receptors would help to explain the generally divergent effects of the alkaloids and the opioid peptides on monocyte and granulocyte function. In general, the nonpeptide opioids are functionally suppressive and the peptides are upregulating. It is of interest that these differences do not seem to exist in the nervous system.

The studies described above clearly show that phagocytic cells of humans and rodents express opioid receptors,

that opioids can bind to the receptors, and that opioids can modulate the function of the cells. Demonstration of effects of the opioids in in vitro assays shows direct action of the drugs and the peptides on these professional phagocytes.

3. Effects of opioids on natural killer cells

A large number of the studies of the effects of opioids on immune function have focused on perturbation of natural killer (NK) cell function. Provocative reports that supported a neuroimmune circuit involving NK cells were published starting in 1984 when Shavit et al. (1984) first found that subcutaneous (sc) injections of morphine into rats suppressed splenic NK cell activity. Depression of NK activity has been demonstrated in heroin abusers (Novick et al., 1989) and in polydrug abusers (Nair et al., 1986). In the latter study, depression of antibody-dependent cellular cytotoxicity was also observed (ADCC) (Nair et al., 1986). In a definitive experiment, reduced NK cell activity, as well as reduced ADCC mediated by NK cells, has recently been shown using human volunteers given morphine intravenously (iv) for 24 h (Yeager et al., 1995). Subcutaneous injection of morphine has also been found to lead to depressed NK cell function in rats (Bayer et al., 1990) and mice (Carr et al., 1994a). Monkey NK cell function has also been reported to be suppressed when morphine was injected sc, on a daily basis, for 2 yr (Carr and France, 1993). It can be concluded that there is strong evidence supporting the conclusion that opioids given in vivo depress NK and ADCC activity.

A number of investigators have addressed the question of whether the depression is due to a direct effect on NK cells or is centrally mediated. Shavit et al. (1986) showed that delivery of morphine into the lateral ventricle of the brain suppressed NK cell activity to the same degree as a single sc injection. The observation that *N*-methyl-morphine, which does not cross the blood–brain barrier, was ineffective when given peripherally to rats, suggested that the depression of NK responses was mediated via opioid receptors in the brain (Shavit et al., 1986). These studies were confirmed and extended by Weber and Pert (1989), who found that morphine acted to depress NK activity via the periaqueductal gray region of the rat brain. Evidence for involvement of sympathetic pathways (Carr et al., 1994b, 1993) or the hypothalamic–pituitary–adrenal (HPA) axis (Freier and Fuchs, 1994) in mediating the suppressive effect of morphine given in vivo on NK cell function in the rat has been published. Carr et al. (1994b, 1993) have shown that pretreatment of mice with *α*-adrenergic receptor antagonists blocked the morphine-induced suppression in NK cell activity. Fecho et al. (1993) however, found that *β*-adrenergic receptor antagonists could not block the suppressive effects of morphine on NK cell activity in the rat. A role for HPA axis involvement in

morphine-induced suppression of NK cell activity was suggested by Freier and Fuchs (1994), who reported that morphine pellet implantation resulted in elevation of serum corticosterone. These authors found that the glucocorticoid receptor antagonist RU 38486 blocked the morphine-induced suppression of NK activity in a dose-dependent manner. However, Band et al. (1992) found that central administration of the *μ*-opioid agonist, DAGO, suppressed NK cell function without elevation of plasma corticosterone. These papers provide strong evidence that the effects of morphine on NK activity are not direct, but are centrally mediated. In vitro studies provide one way to confirm or reject this conclusion.

There is little evidence that morphine can modulate NK function by direct action on these cells. Mathews et al. (1983) reported that *β*-endorphin added to human PBMCs enhanced NK cell activity, but morphine was inactive. Variable stimulatory activity of *β*-endorphin and met–enkephalin was observed by other investigators (Puente et al., 1992; Oleson and Johnson, 1988). Ochshorn-Adelson et al. (1994) reported that naloxone did not affect NK cell cytotoxicity when added to human PBMCs. Similarly, other investigators have failed to find an effect of morphine in vitro on NK cell activity in rodents (Freier and Fuchs, 1994). It is interesting that, as with the phagocytic cells, the endogenous opioids seem to have an upregulating rather than a down regulating effect. Further, in vitro effects have been demonstrated with some of the peptides, but not with morphine. The discordance between peptide and alkaloid data indicates that if the receptor is the same, downstream signalling events are different. The paucity of studies reporting an in vitro effect of morphine on NK cell activity and the substantial literature showing that effects on NK activity are centrally mediated support the conclusion that the alkaloid is not acting directly on the NK cell.

4. Effects of opioids on B cells

The first study to show an effect of morphine on immune function was that of Lefkowitz and Chiang (1975), who reported that morphine injection reduced the number of antibody-forming cells in the mouse spleen following immunization with sheep red blood cells. The effect of antagonists was not reported. Subsequently, Bryant et al. (1988) found that implantation of a 75-mg morphine slow-release pellet reduced the mitogenic responses of splenic B cells to bacterial lipopolysaccharide and this has been confirmed using an injection dosing regimen (Bhargava et al., 1994). Bussiere et al. (1992a) reported that 75-mg morphine pellets also inhibited induction of antibody-forming cells to sheep red blood cells in a variety of mouse strains and showed that suppression was naltrexone-inhibitable in some mouse strains, but not others, and this observation has been confirmed (Pruett et al., 1992). Further, Bussiere et al. (1992a,b) showed that both mice

with defects in response to LPS, and beige mice, which lack NK cells, had suppressed antibody responses when treated with morphine, however, mice which lack μ-opioid receptors did not (Bussiere et al., 1992a). Formation of an antibody response to sheep red blood cells requires interaction of macrophages, T cells, and B cells. Thus, depression of the capacity to produce antibody does not necessarily mean that the effect of the drug is on B cells. Morphine could be affecting any of the 3 cell types. In fact, Bussiere et al. (1993) found that antibody responses could be restored by adding normal macrophages or IL-1, IL-6 or Interferon-γ (IFN-γ), cytokines which are produced by macrophages or which activate macrophages, suggesting that the morphine-induced suppression is due in part to a deficit of macrophage activity. Weber et al. (1987) also found that morphine inhibited serum antibody responses to a T-dependent, but not to a T-independent antigen, suggesting that morphine did not affect B-cell function.

The question has also been raised as to whether there is a direct effect of morphine on the cells involved in antibody formation, or if these effects are also mediated, for example by the HPA axis (Pruett et al., 1992). Taub et al. (1991) has shown that morphine, as well as the κ-opioids U50,88H and U69,593, inhibited antibody formation to SRBCs when added to mouse spleen cells in vitro. Eisenstein et al. (1995) confirmed that both morphine and U50, 488H could inhibit in vitro antibody responses, showing that the effects of the drugs are directly on immune cells and suppression is not mediated by the HPA axis. In this study, marked differences in mouse strain susceptibility to the effects of morphine and the κ agonist were demonstrated, raising the possibility that failure by others to demonstrate in vitro effects is attributable to the mouse strain used (Eisenstein et al., 1995). The suppressive effects of a κ agonist on the plaque-forming cell antibody response, whether given in vivo or in vitro, have been confirmed by Radulovic et al. (1995) in a rat model. Guan et al. (1994) examined the effect of U50,488H, when added in vitro, to T cell- or macrophage-enriched fractions of normal mouse spleens and found that the opioid inhibited activity of both of these cell types. Effects on B cells were not tested directly, but were not ruled out by these experiments. A direct effect of morphine on murine splenic B-cell proliferation to mitogen has also been reported (Thomas et al., 1995a). Interestingly, for antibody formation, endogenous peptides have also been reported to be suppressive (Johnson et al., 1982; Morgan, 1996).

5. Effects of opioids on T cells

There is considerable evidence that morphine given in vivo modulates T-cell function. McDonough et al. (1980) showed that heroin addicts have reduced numbers of cells that can rosette with sheep erythrocytes, a measure of T cells, and that treatment with naloxone restored binding to

a normal frequency. Studies in addicts are complex, as many are polydrug abusers. Donahoe et al. (1986) found that simultaneous abusers of heroin and cocaine did not have depressed T-cell rosettes, suggesting that the two drugs have cancelling effects. Heroin addicts have also been reported to have reduced CD4^{+} cells and elevated CD8^{+} cells (Donahoe et al., 1987) in one study, but no change in T-lymphocyte cell subsets in another study (Novick et al., 1989). Whether the observed alterations in cell numbers are due to drug abuse or to other life-style alterations in abusers cannot be easily tested. Other parameters of T-cell function in response to drug use have not been studied in humans.

Studies in experimental animals have allowed a clearer delineation of a cause-and-effect relationship between opioid administration and alteration in T-cell function. Morphine has been shown to alter the ratio of CD4^{+} and CD8^{+} T-lymphocytes in both the spleen (Arora et al., 1990) and thymus (Arora et al., 1990; Freier and Fuchs, 1993) of mice implanted with a 75-mg morphine pellet, as well as the peripheral blood of monkeys which received daily injections of morphine for 2 yr (Carr and France, 1993). On a functional level, morphine administration has been shown to inhibit the capacity to develop a delayed-type hypersensitivity response (a measure of cellular immunity) to attenuated mycobacteria in rats (Pellis et al., 1986), to picryl chloride in mice (Bryant and Roudebush, 1990), and to 2,4-dinitrofluorobenzene in pigs (Molitor et al., 1992). Morphine has also been reported to inhibit induction of cytotoxic T-cell responses in mice following immunization with allogenic spleen cells (Carpenter and Carr, 1995; Carpenter et al., 1994). A commonly used measure of T-cell function is the capacity to respond to the mitogen, Con A. In mice, Ho and Leung (1979) observed a depression in the proliferative response of lymphocytes to Con A from mice addicted to morphine. Bryant et al. (1987) found that administration of morphine via a slow-release pellet in mice decreased splenic responses to Con A but, in contrast to the previous report, observed that the depression in T-cell activation disappeared as mice became tolerant. Similar results to those of Bryant were observed in monkeys given daily injections of morphine (Chuang et al., 1993). Studies in Bayer's laboratory showed that rats given a single sc injection of morphine had depressed proliferative capacity of blood mononuclear cells in response to Con A (Bayer et al., 1990). Using this paradigm, proliferation of spleen cells was unaltered (Bayer et al., 1990). Lysle et al. (1993) examined blood, spleen, and mesenteric lymphnode responses after a single sc injection of various doses of morphine in rats and confirmed the suppression of mitogenic stimulation in blood mononuclear cells, but they also found suppression of spleen cell responses, with unaltered mesenteric lymphocyte responses.

A number of investigators have examined mechanisms by which morphine induces suppression of T-cell activi-

ties. Flores et al. (1994) showed that the depression of Con A responses in peripheral blood cells of rats was not dependent on glucocorticoids or on pituitary factors, as neither adrenalectomy nor hypophysectomy abrogated the suppression. However, part of the suppression was attributable to adrenal-mediated decreases in numbers of circulating lymphocytes (Flores et al., 1995). Fecho et al. (1994, 1995) attributed the depression in Con A responsiveness of rat splenocytes to release of splenic macrophage-derived nitric oxide. In vitro use of a nitric oxide inhibitor to treat spleen cells taken from rats given morphine abolished suppression (Fecho et al., 1994). Fecho et al. (1993) has also presented experiments to support involvement of the β-adrenergic system in morphine-induced suppression of spleen cells to Con A or to another T-cell mitogen, PHA. Interestingly, they found no effect of β-adrenergic antagonists on morphine-induced suppression of mitogenic responses in peripheral blood cells (Fecho et al., 1993). Studies using opioids which do not pass the blood–brain barrier supported a conclusion that the effects of morphine on splenic T cells were mediated via central opioid receptors (Fecho et al., 1996b). In addition, adrenalectomy, alone or in combination with drugs that block the sympathetic nervous system, pointed to the conclusion that the sympathetic system was involved in the suppression by morphine of splenic responses to PHA, but that the adrenocortical system mediated suppression by morphine of T-cell responses in the blood to Con A or PHA (Fecho et al., 1996a). The mechanism by which the sympathetic nervous system influences nitric oxide levels has not been addressed. Sei et al. (1991) found that morphine inhibited the increase in cytoplasmic free Ca^{2+} induced by mitogens in mouse splenic $CD4^+$ cells through a glucocorticoid-dependent mechanism, as adrenalectomy abolished the effect. Thus the results of the three groups that have examined the role of the adrenal gland in mediating the morphine-induced suppression of T-lymphocyte function are not completely consonant. However, different rat strains were used by two of the laboratories (Flores et al., 1995; Fecho et al., 1993), and the other study was done in mice (Sei et al., 1991), which might account for the discrepancies.

It is interesting that the literature, as reviewed above, suggests that the effect of morphine on T-cell responses may be indirect. There are several studies which failed to find an effect of the drug when it was added to T cells in vitro (described below) and only a few papers where direct effects of the drug were found. This is a surprising situation, since among the first published effects of morphine on immune cells was the observation of Wybran that morphine added to human peripheral blood lymphocytes inhibited T rosettes (Wybran et al., 1979). Inhibition of T rosette formation has been confirmed by two groups using human cells (de Carolis et al., 1984; Donahoe et al., 1987; Donahoe et al., 1988) and has also been shown to occur with monkey cells (Donahoe et al., 1988). A weak sup-

pressive effect of heroin on Interleukin-2 (IL-2) production by murine spleen cells was observed at some doses, but not others, and the effect of antagonists was not tested (Thomas et al., 1995b). Selective suppression of in vitro proliferative responses of two mouse strains to a panel of three mitogens has been reported (Eisenstein et al., 1991). Further, the κ-opioid agonist, U50,488H, has been reported by Guan et al. (1994) to suppress the capacity of T cells to participate in an in vitro antibody response to sheep erythrocytes. Of interest is the fact that κ-binding sites have been clearly demonstrated in a very immature murine thymoma cell line (Lawrence and Bidlack, 1993; Lawrence et al., 1995). Furthermore, κ-opioid receptor mRNA has been detected in the same cell line (Belkowski et al., 1995c) and sequence analysis indicated that this message was identical to the murine brain κ-opioid receptor (Belkowski et al., 1995b). In contrast to the immunosuppressive effects of morphine reviewed so far, there are two reports in the literature of stimulation of T-cell responses to mitogens by morphine. Bocchini et al. (1983) found that morphine and naloxone both enhanced human PBMC responses to PHA, and Bidlack and Hemmick (1990) reported that high doses of morphine enhanced PHA responses of rat lymph node cells, and naloxone did not block the effect.

There are several papers in which no in vitro effect of morphine was observed on T-cell function. Bayer et al. (1992) could not demonstrate an inhibitory effect of morphine added into rat blood mononuclear cell cultures stimulated with Con A, except at very high doses that were not blocked by naloxone, and Fecho et al. (1996b) obtained similar results using rat spleen or blood lymphocytes stimulated with Con A or PHA. Similarly, Sei et al. (1991) did not find an in vitro effect of morphine on Ca^{2+} flux in murine splenocytes stimulated with Con A. Chuang et al. (1993) failed to find a direct effect of morphine on monkey peripheral blood cells stimulated with T-cell mitogens. The negative results are not only surprising in light of the paper of Wybran et al. (1979), but also because naloxone has been shown to bind to human T-lymphocytes (Madden et al., 1987; Mehrishi and Mills, 1983) and to rat splenocytes activated by mitogens (Ovadia et al., 1989). Morphine has also been shown to bind to PHA- and IL-1-stimulated mouse thymocytes (Roy et al., 1991a). These results seem to suggest that morphine binding sites in rodents may only be observed after cell activation.

6. Summary

There is incontrovertible evidence that morphine inhibits the function of NK cells, B cells, T cells, and phagocytic cells when it is given in vivo. Direct effects of the drug have also been demonstrated in vitro on phagocytic cells. It is less clear whether morphine has a direct effect on NK cells or B cells. There is strong evidence that

morphine can directly modulate receptor expression on T cells, but consistent alteration of other functional parameters in vitro has been difficult to demonstrate. Evidence for a direct effect of κ agonists on murine T cells exists.

Future studies that better delineate opioid receptor distribution and expression on cells of the immune system will enhance our understanding of the parameters which influence functional changes induced by morphine and other opioids. Responsiveness to opioids may depend on the state of cell activation, which can influence receptor expression. Alternatively, various immune cells may express more than one type of opioid receptor, and functional alterations may depend upon the relative activation of one type as compared with another. As endogenous peptides seem to have opposite functional effects from nonpeptide agonists in a number of cases, and immune cells may even synthesize such peptides (Zurawski et al., 1986; Kuis et al., 1991), the interactions of exogenous and endogenous opioids may result in complex alterations in immune cell function. The presence of unrecognized endogenous opioid peptides that can antagonize the alkaloids or synthetic opioid peptides may account for the variable effects of morphine seen in different studies of T-cell function.

The consistent observation of the suppressive effects of morphine given in vivo provide the potential for increased susceptibility to infectious diseases, regardless of whether the mechanism of the suppression is directly on immune cells or through the perturbation of other systems in the body which ultimately down regulate immune function.

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The μ-opioid receptor subtype is required for the anorectic effect of an opioid receptor antagonist

Jiaping Zhang [a,1], Andrea Frassetto [a,1], Ruey-Ruey C. Huang [a], Julie Z. Lao [a],
Alexander Pasternak [b], Sheng-ping Wang [c], Joseph M. Metzger [c],
Alison M. Strack [c], Tung M. Fong [a], Richard Z. Chen [a,*]

[a] Department of Metabolic Disorders, Merck Research Laboratories, P.O. Box 2000, RY80M-213, Rahway, NJ 07065, USA
[b] Department of Medicinal Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA
[c] Department of Pharmacology, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA

Abstract

A diaryl ether derivative, (6-(4-{[(3-methylbutyl)amino]methyl}phenoxy)nicotinamide, was prepared and investigated for its biochemical properties at cloned opioid receptors and its pharmacological effects on animal feeding. The compound displaced [^{3}H]DAMGO binding of human μ-opioid receptor, [^{3}H]U-69593 of human κ-opioid receptor, and [^{3}H]DPDPE of human δ-opioid receptor with IC_{50} values of 0.5±0.2 nM, 1.4± 0.2 nM, and 71±15 nM, respectively. The compound also potently inhibited [^{3}H]DAMGO binding of cloned mouse and rat μ-opioid receptors ($IC_{50} \approx 1$ nM), and acted as a competitive antagonist in a cAMP functional assay using cultured cells expressing human or mouse μ-opioid receptors. Following a single oral administration in diet-induced obese mice (at 10 or 50 mg/kg) or rats (at 1, 3, or 10 mg/kg), the compound caused a dose-dependent suppression of acute food intake and body weight gain in both species. Importantly, the anorectic efficacy of the compound was mostly diminished in mice deficient in the μ-opioid receptor. Our results suggest an important role for the μ-opioid receptor subtype in animal feeding regulation and support the development of μ-selective antagonists as potential agents for treating human obesity. © 2006 Elsevier B.V. All rights reserved.

Keywords: Opioid receptor antagonist; Knockout mice; Food intake; Body weight

1. Introduction

There is a considerable amount of evidence supporting that modulation of the endogenous opioid system, which consists of distinct subtypes of receptors (μ, δ, and κ; all members of the G protein-coupled receptor superfamily) and opioid peptides (β-endorphin, the enkephalins, and the dynorphins), affects feeding in mammals. Opioid receptors and peptides are expressed in brain regions that are important for feeding regulation, and elevated levels of endogenous opioid peptides were observed in obese or fasting animals (Ferguson-Segall et al., 1982; Herve and

Fellmann, 1997; Margules et al., 1978; Welch et al., 1996). Opioid receptor agonists, including morphine and endogenous opioid peptides β-endorphin and dynorphin, have been shown to increase food intake (Glass et al., 1999; Reid, 1985; Sanger and McCarthy, 1981), while the opposite effect has been demonstrated for opioid receptor antagonists in a variety of species (Billington et al., 1985; de Zwaan and Mitchell, 1992; Deviche and Wohland, 1984; Glass et al., 1999; Yeomans and Gray, 2002). Significantly, the inhibition of long-term body weight gain has been reported in rats following chronic administration of naltrexone (Glass et al., 2002; Marks-Kaufman et al., 1984; Shaw, 1993) or LY255582 ((3R,4R)-1-(S)-3-hydroxy-3-cyclohexylpropyl-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidine) (Shaw, 1993; Statnick et al., 2003), two non-selective and structurally distinct opioid receptor antagonists. These observations support

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* Corresponding author. Tel.: +1 732 594 4064; fax: +1 732 594 3337.
  E-mail address: richard_z_chen@merck.com (R.Z. Chen).
[1] These authors contributed equally to this work.

the proposal that blockade of the central opioid pathway by opioid receptor antagonists may represent a potential means to treat human obesity (Statnick et al., 2003; Yeomans and Gray, 2002).

Pharmacological studies using putative subtype-selective opioid receptor ligands or gene-specific antisense oligonucleotides have so far suggested a minimal role for the δ-opioid receptor subtype in the regulation of feeding behavior (Arjune et al., 1991; Hadjimarkou et al., 2004; Koch and Bodnar, 1994; Kotz et al., 1993). On the other hand, µ- and κ-opioid receptors seem to be an integral part of brain networks that control energy balance, especially the reward aspect of feeding (Barton et al., 1996; Bodnar, 2004; Hadjimarkou et al., 2004; Koch and Bodnar, 1994). Although there have been studies suggesting a predominant role for the µ-opioid receptor in mediating the effects of opioids on feeding, the interpretation of these data is complicated by the fact that the in vivo selectivity of the pharmacological tools has not yet been firmly established. The genes that encode the three opioid receptor subtypes have been cloned, and all three genes have been disrupted in mice (Kieffer and Gaveriaux-Ruff, 2002). These mutant mice have provided a unique tool to study the physiological role of each opioid receptor (Kieffer and Gaveriaux-Ruff, 2002). To further define the role of µ-opioid receptor signaling in feeding, we compared the effects of a potent, non-selective opioid receptor antagonist on acute food intake in wild-type mice vs. those in mice lacking the µ-opioid receptor (Schuller et al., 1999). Our results show that the efficacy of the antagonist at reducing food intake observed in wild-type mice was mostly diminished in the µ-opioid receptor knockout animals, thus supporting that signaling through the µ-opioid receptor plays a major role in mediating the effects of opioids on feeding.

2. Materials and methods

2.1. Materials

Compound A (6-(4-{[(3-methylbutyl)amino]methyl}phenoxy) nicotinamide; Fig. 1A) was synthesized internally as described in a patent application (Blanco-Pillado et al. PCT Patent Application WO 2004/026305 A1). [³H]DAMGO (Tyr–D-Ala–Gly–N-methyl–Phe–Gly-ol; 42.5 Ci/mmol), [³H]U-69593 (N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-8-yl]-benzeneacetamide; 47.5 Ci/mmol) and [³H]DPDPE (D-Pen²-D-Pen⁵-enkephalin; 44.0 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All cell culture and transfection reagents, unless indicated otherwise, were obtained from Invitrogen (La Jolla, CA).

2.2. Cell lines and cell membrane preparation

Cell lines stably expressing human, mouse or rat µ-opioid receptors, or human κ- or δ-opioid receptors were established by cloning the respective receptor cDNA into pCI-Neo (Promega, Madison, WI) and transfecting the constructs into Chinese hamster ovary (CHO) cells (R.Z. Chen et al., unpublished). For membrane preparation, cells were collected and lysed in ice-cold buffer containing 10 mM Tris–HCl (pH 7.2), 1 mM EDTA and 1× protease inhibitor cocktails (Sigma-Aldrich) and homogenized
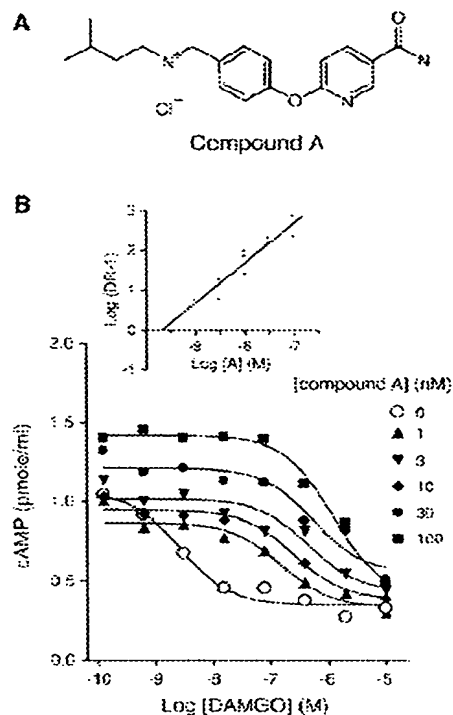

Fig. 1. (A) Structure of compound A (6-(4-{[(3-methylbutyl)amino]methyl} phenoxy)nicotinamide). (B) Effects of compound A on DAMGO-induced suppression of adenylate cyclase activity in cells stably expressing human µ-opioid receptor. 10 µM of forskolin was included to boost the cellular cAMP level. Inset: Schild analysis of compound A, n = 3.

using a motor-driven homogenizer. Following a low speed centrifugation (700 ×g for 10 min), the supernatant was collected and centrifuged again at 38,000 ×g for 20 min. The pellet was subsequently re-suspended by passing through a 25 G needle a few times in membrane storage buffer containing 50 mM Tris–HCl (pH 7.2), 2.5 mM EDTA, 5 mM MgCl₂, 0.29 M sucrose and 1× protease inhibitor cocktails, and stored at −80 °C in aliquots. Protein concentrations were determined using the Coomassie-Plus Protein Assay Reagent (Pierce, Rockford, IL).

2.3. In vitro binding and functional assays

Affinities (IC₅₀) of compounds for cloned opioid receptors were measured by their abilities to displace radio-labeled ligands using cell membranes prepared from cultured cells and scintillation proximity assay (SPA) beads that bind to cell membranes (Catalog No. RPNQ0001; GE Healthcare Life Sciences, Piscataway, NJ). Assays were carried out in 200 µl binding buffer (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 2 mM MgCl₂) in 96-well plates. Cell membranes were re-suspended in binding buffer to final concentration of 0.02–0.2 mg protein/ml and mixed with reconstituted SPA beads to a final concentration of 0.25 mg/ ml. To each well, 148 µl of the above mixture, 2 µl of the cold compound (to 10⁻¹⁰ to 10⁻⁵ M final concentration) and 50 µl of the respective radioligand dilution (Table 1; to the following final concentration: [³H]DAMGO, 1 nM; [³H]U-69593, 0.5 nM; [³H]

DPDPE, 2 nM) were added. Following 1 h incubation at room temperature, the radioactivity bound to the cell membranes was measured on a scintillation counter.

Cellular cAMP levels were measured to evaluate the functional activity of compounds at μ-opioid receptors using the SPA cAMP Screening System (Catalog No. RPA556, GE Healthcare) in 96-well OptiPlates (Catalog No. 6005190, PerkinElmer). Harvested cells were re-suspended at approximately 2.5×10^6 cells/ml in assay medium containing Earle's Balanced Salt Solution (EBSS, pH 7.4) supplemented with 25 mM Hepes, 5 mM $MgCl_2$ and 0.1% BSA. A standard curve of known cAMP concentrations was constructed and added into the designated wells. Cells (50 μl, $\sim 10^5$ cells) were transferred to the remaining wells, to give a final volume of 100 μl containing 10^{-10} to 10^{-5} M DAMGO, 10 μM forskolin (to elevate cellular cAMP level) and 200 μM 3-isobutyl-1-methylxanthine (to inhibit phosphodiesterases). The plate was incubated at 37 °C for 30 min and the reaction was terminated by boiling for 3 min. The accumulation of cAMP in 15 μl lysate was measured according to manufacturer's instructions.

2.4. In vivo studies

For feeding studies in mice, male wild-type and μ-opioid receptor knockout (Schuller et al., 1999) C57BL/6 mice were bred and maintained at Taconic Farms through special arrangements and were imported to Merck when reaching 4–6 weeks of age. At the time of dosing, the mice were approximately 10 months old and had been individually caged and maintained on a high-fat diet (45% kcal from fat; D12451, Research Diets, New Brunswick, NJ) for approximately 9 months. The mice were culled to a relatively narrow body weight range (mean±S.E.M.: wild-type, 51.4±0.7 g; knockout, 55.9±1.0 g) and assigned randomly to individual experimental groups (n=8–10 per group). Rat feeding studies were carried out using male diet-induced obese (DIO) CD rats (Charles River Laboratories, Wilmington, MA) that had been individually caged and maintained on a medium high-fat diet (32% kcal from fat; D12266B, Research Diets). At the time of the experiment, the rats (n=5–7 per group) were approximately 4 months old weighing approximately 570 g. The compound was administered by oral gavage using 5% Tween 80 and 0.5% methylcellulose (mouse) or 10% Tween 80 (rat) as the vehicle approximately 1 h before the onset of dark cycle. All animal protocols used in the study were in accordance with the NIH

Table 1
IC_{50} values for binding of compound A and a few control compounds to human (hu)/mouse (m)/rat (r) μ-, human κ- and human δ-opioid receptors

	IC_{50}, nM				
	μ (hu)	μ (m)	μ (r)	κ	δ
Compound A	0.5±0.2	1.1±0.5	2.3±0.6	1.4±0.2	71±15
DAMGO	1.7±0.2	1.8±0.3	0.8±0.1	n.d.	n.d.
Nalmefene	1.0±0.2	0.9±0.2	0.5±0.1	0.4±0.1	17±2.0
Naloxone	2.8±1.0	3.0±0.6	2.1±0.2	6.5±1.3	138±12

Data (mean±S.E.M.) are derived from three to five independent binding experiments performed in duplicate as described in Materials and methods, using [³H]DAMGO (μ; final concentration, 1 nM), [³H]U-69593 (κ; 0.5 nM), and [³H]DPDPE (δ; 2 nM) as the indicated radioligand. n.d., not determined.

Guide for Care and Use of Laboratory Animals and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (IACUC) at Rahway, New Jersey.

2.5. Statistical analysis

Data from in vitro binding or functional assays were analyzed by linear or non-linear regression using the PRISM software (version 4, GarphPad, San Diego, CA). A two-way analysis of variance (ANOVA) was performed to determine the differences in food intake and body weight changes in mice between vehicle- and compound-treated groups and between wild-type and μ-opioid receptor knockout genotypes. A two-way ANOVA was also used to determine the differences in food intake in rats between vehicle- and compound-treated groups and between different time points, and the differences in body weight changes in rats between vehicle- and compound-treated groups were analyzed by one-way ANOVA. The Dunnett's test was used as a post-hoc analysis for multiple comparisons. In all cases, significance was at $P<0.05$.

3. Results

3.1. In vitro binding and functional studies of compound A

The structure of compound A is shown in Fig. 1A. In radioligand competition binding assays, compound A displaced [³H] DAMGO binding of cloned human, mouse or rat μ-opioid receptors with IC_{50} values of 0.5±0.2 nM, 1.1±0.5 nM, and 2.3± 0.6 nM, respectively (Table 1). The compound also displaced [³H] U-69593 binding of human κ-opioid receptor with a potent IC_{50} value (1.4±0.2 nM), but was significantly less potent at inhibiting [³H]DPDPE binding of human δ-opioid receptor ($IC_{50}=71 \pm$ 15 nM). Compound A did not show any significant binding affinity in the low-micromolar range for several other G protein-coupled receptor subfamilies, such as those for serotonin, melanocortins, cannabinoids, and neuropeptide Y (data not shown). The properties of compound A in affecting the signaling of μ-opioid receptors were assessed in a functional cAMP-binding assay using DAMGO to stimulate CHO cells stably express human or mouse μ-opioid receptors. Agonist-induced activation of the μ-opioid receptor is coupled through the inhibitory $G_{i/o}$ proteins, leading to the suppression of adenylyl cyclase (Eguchi, 2004). As shown in Fig. 1B for cells expressing mouse μ-opioid receptor, in the presence of forskolin (10 μM, to elevate cellular cAMP levels), increasing concentrations of DAMGO caused decreases in intracellular cAMP levels. The addition of compound A in the cell culture led to a dose-dependent rightward shift of the DAMGO concentration response curve. Interestingly, the basal (forskolin alone) cAMP level seemed to be elevated when a high concentration of compound A was added (Fig. 1B), a phenomenon that was also observed when the compound was assayed alone (data not shown). Schild analysis produced a slope of 0.997 confirming competitive antagonism, and pA_2 of 9.7 in agreement with the IC_{50} value (Fig. 1B, inset). Similar data were obtained using cells expressing human μ-opioid receptor (data not shown). Thus, compound A is

a competitive antagonist, which may possess a weak inverse agonist activity at the μ-opioid receptor.

3.2. Feeding studies of compound A

The acute effects of compound A on food intake and body weight regulation were first determined in DIO rats. The apparatus used in the rat studies allowed measurement of food intake as frequently as every 5 min through a computerized process. Fig. 2A shows cumulative food intake at hourly intervals following a single oral administration of either vehicle or compound A at 1, 3, or 10 mg/kg. The compound showed an early onset effect and suppressed food intake throughout the dark cycle. Overnight (18 h) food intake was significantly reduced for all three compound-treated groups (−35%, −50%, and −80% for 1, 3, or 10 mg/kg, respectively) as compared to vehicle-treated group. No signs of taste aversion were observed following compound administration. While animals in the vehicle group gained approximately 5 g of body weight following a dark cycle, which is within a typical range for animals kept on a 12-h light/dark cycle with free access to food, animals in the compound-treated groups lost 5 g or more ($P<0.01$). There were no significant differences in water consumption between vehicle-treated animals and those dosed with compound A (data not shown).

Mice deficient in the μ-opioid receptor (Schuller et al., 1999) were used to assess the role of the receptor in mediating the

Fig. 3. Effects of compound A on 4-h and overnight (18 h) food intake and overnight body weight (BW) change in wild-type (WT) and μ-opioid receptor knockout (KO) mice. At the time of dosing, WT and KO mice weighed approximately 52 and 56 g, respectively. *$P<0.05$, **$P<0.01$ vs. vehicle. Values shown are the mean±S.E.M. ($n=8$–10 per group).

anorectic effect of compound A, with age- and body weight-matched wild-type mice as controls. As shown in Fig. 3, a single oral dose of compound A at 10, or 50 mg/kg suppressed a 4-h food intake by 49% and 86% (both $P<0.01$), respectively, in the wild-type mice, and 31% (n.s.) and 34% ($P<0.05$), respectively, in the knockout mice. The same doses suppressed an 18-h food intake by 29% ($P<0.05$) and 70% ($P<0.01$), respectively, in the wild-type mice, but were not efficacious in the knockout mice. While wild-type mice dosed with 50 mg/kg of compound A lost significantly more body weight overnight (18 h) than the vehicle control (2.2±0.3 g vs. 0.2±0.3 g, $P<0.01$), there were no differences in 18-h body weight changes in the knockout mice between vehicle and those dosed with compound A. Vehicle-treated groups of either genotype showed no differences in food intake or overnight body weight change. Thus, the efficacy of compound A at suppressing acute food intake and body weight gain was substantially blunted in μ-opioid receptor knockout mice.

4. Discussion

The purpose of this study was to examine the role of the μ-opioid receptor in mediating the effects of opioids on feeding. We therefore used μ-opioid receptor knockout mice and compared the effects of an opioid receptor antagonist on feeding in the mutant mice with those in wild-type animals. We found that a non-selective opioid receptor antagonist (compound A; Fig. 1A, Table 1) displayed potent efficacies at suppressing acute food intake and body weight gain in wild-type animals, but lost most of its efficacy in μ-opioid receptor knockout mice (Fig. 3). Our results provide a strong piece of evidence suggesting that signaling through the μ-opioid receptor is necessary for the effects of opioid receptor antagonists on animal feeding.

It remains unclear what was responsible for the residual and transient efficacy of compound A, especially at 50 mg/kg, in the
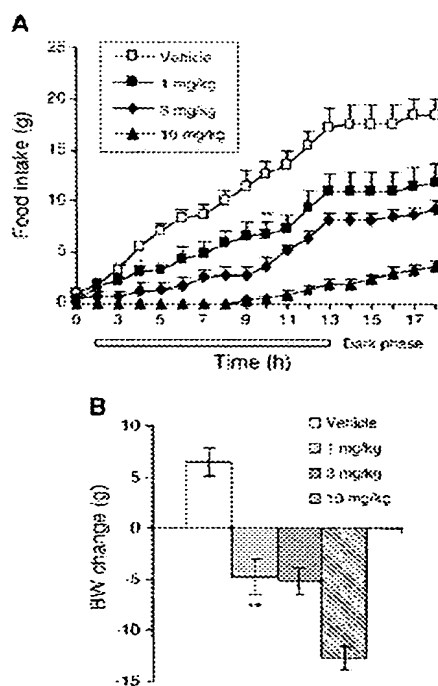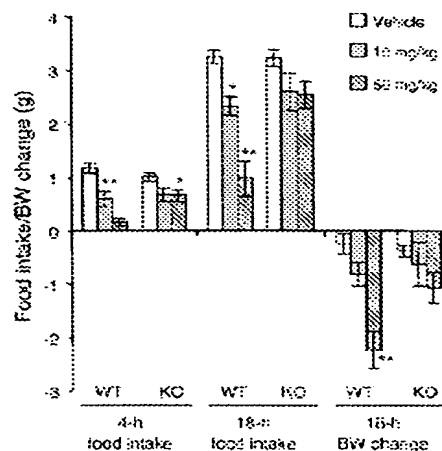

Fig. 2. Effects of compound A on (A) food intake (shown are cumulative food intake at hourly intervals) and (B) overnight (18 h) body weight (BW) change. At the time of dosing, the rats weighed approximately 570 g. *$P<0.05$, **$P<0.01$ vs. vehicle. Values shown are the mean±S.E.M. ($n=5$–7 per group).

knockout mice (Fig. 3). Since compound A also showed a high binding affinity for the κ-opioid receptor ($IC_{50} \approx 1$ nM) and a significant binding affinity for the δ-opioid receptor ($IC_{50} \approx 70$ nM), it is possible that signaling through either or both receptors could play a role. However, if this were the case, contributions from either or both receptors would appear to be marginal compared to that from the μ-opioid receptor. A minimal role for the δ-opioid receptor in feeding as relative to the μ-opioid receptor is consistent with the results of previous pharmacological studies using putative subtype-selective opioid receptor antagonist and/or gene-specific antisense oligonucleotides in various feeding paradigms (Arjune et al., 1991; Hadjimarkou et al., 2004; Koch and Bodnar, 1994; Kotz et al., 1993). However, previous studies also suggested a significant role for the κ-opioid receptor in mediating the effects of opioids on feeding (Barton et al., 1996; Bodnar, 2004; Hadjimarkou et al., 2004; Koch and Bodnar, 1994), which is in contrast to the implication of our results. The discrepancy may be related to our lack of knowledge of the true in vivo selectivity or specificity of the pharmacological tools employed in these studies. Alternatively, signaling through different opioid receptor subtypes may intersect at various points along the pathways and removal of one receptor, as in μ-opioid receptor knockout mice, may affect the signal tone mediated by other receptors. For example, in vivo existence of μ/δ or κ/δ heterodimers/oligomers has been suggested in several recent studies using bivalent ligands, and their pharmacological activation or inhibition was shown to have significant physiological effects (Bhushan et al., 2004; Daniels et al., 2005a,b). It is conceivable that if signaling through these receptor complexes were to play a major role in mediating the effects of opioids on feeding, the absence of one receptor could also abolish the activities of agonists or antagonists directed at other receptors. In this regard, the diminished efficacy of a non-selective opioid receptor antagonist in μ-opioid receptor knockout mice, which nevertheless supports a prerequisite role for the μ-opioid receptor in mediating the effects of opioids on feeding, does not necessarily rule out a role for the κ- or δ-opioid receptors.

A recent study of μ-opioid receptor knockout mice generated by another group of researchers (Matthes et al., 1996) provided evidence that the μ-opioid receptor plays a specific role in energy homeostasis (Tabarin et al., 2005). The study showed that the knockout mice did not differ from the wild-type in ad libitum food intake either on a regular diet or on a high-fat diet, but on a high-fat diet the knockout mice gained less weight and less fat due to an apparent increase in the use of fat as the main fuel source. Similarly, we did not observe any differences in food intake or body weight between wild-type and μ-opioid receptor knockout mice maintained on a regular chow diet. On a high-fat diet, however, we observed that the knockout mice showed a trend toward gaining more weight than wild-type controls (R.Z. Chen et al. unpublished), which is in contrast to the observations by Tabarin et al. (2005). The reason for this discrepancy is unclear.

In conclusion, our results support the development of antagonists selective for the μ-opioid receptor as potential anti-obesity pharmaceutical agents, which may have a simple mechanism of action and thus possess reduced risk of unwanted effects.

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Methylnaltrexone Progenics
Fabrizio De Ponti

Address
Department of Pharmacology
University of Bologna
Via Irnerio 48
40126 Bologna
Italy
Email: deponti@biocfarm.unibo.it

Progenics is developing methylnaltrexone (MNTX), an opioid antagonist licensed from UR Labs and the University of Chicago, for the potential treatment of the side effects of opioid pain therapy, such as constipation and post-operative bowel dysfunction. MNTX was discovered at the University of Chicago and subsequently licensed by UR Labs. In October 2001, Progenics entered into an agreement with UR Labs to obtain exclusive worldwide rights to the drug [423791]. MNTX is in phase II trials, with phase III studies expected to begin in 2002 [423791]. In October 2001, a double-blind, randomized, phase II study evaluating sc MNTX in cancer patients for the treatment of opioid-induced constipation was initiated at the University of Chicago Medical Center [423791]. In December 2001, the company stated that it was preparing to initiate phase IIb trials of the compound in opioid-induced constipation and post-operative bowel dysfunction [423791], [432507]. These trials were initiated in February 2002 [440995].

Introduction

In patients taking narcotics for the relief of chronic pain or other medical indications, constipation is a common and sometimes severe side effect, which is not adequately controlled by currently available drugs [423917], [428618], [434583]. The effects of exogenous opioids on gastrointestinal motility are mediated by receptors located both centrally and peripherally (enteric neurons) [434584], whereas analgesia is mediated by receptors located in the brain and in the spinal cord. Although the relative contribution of central and peripheral receptors in mediating opioid-induced constipation in humans is still unclear, circumstantial evidence suggests an important role is played by peripheral receptors [423923]; hence, the rationale for using a peripheral, selective, opioid antagonist to obtain relief from opioid-induced constipation, while preserving the desired central analgesic effect. MNTX is an example of such an antagonist. Other undesirable side effects of opioids that may be sensitive to blockade by peripheral opioid antagonists include delayed gastric emptying, nausea and vomiting, urinary retention and pruritus [423923], [428618]. These side effects are also potential therapeutic targets for MNTX, but this evaluation will focus only on opioid-induced constipation.

Synthesis and SAR

MNTX is a quaternary derivative of the opioid antagonist naltrexone. Antagonists such as naloxone and naltrexone, as tertiary compounds, are fairly lipid-soluble and cross the blood-brain barrier easily. Addition of the methyl group at the tertiary amine forms a quaternary compound with greater

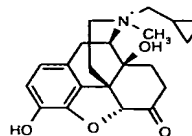| Field	Value
Originator	University of Chicago
Licensees	Progenics Pharmaceuticals Inc, UR Labs Inc
Status	Phase II Clinical
Indication	Constipation
Action	Opioid receptor antagonist
Synonyms & Analogs	MNTX, methylnaltrexonium
CAS	Morphinanium, 17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxy-17-methyl-6-oxo-, (5α)-
	Registry No: 83387-25-1

polarity and lower lipid solubility. For example, following ip injection of MNTX (10 mg/kg) in rats, the concentrations of the drug in several brain areas are only 5 to 7% of the concentrations after the same dose of naltrexone [434585].

An important aspect to be assessed when designing quaternary compounds is whether they maintain high affinity for the opioid receptors and whether or not their metabolites can cross the blood-brain barrier [434586]. With regard to the former, quaternarization of opiate antagonists generally greatly diminishes their affinity for opioid receptors; MNTX is reported to possess only 1.3 to 3.9% of the antagonist activity of naltrexone *in vitro* [434586]. This is a potential disadvantage of the compound with respect to another peripherally selective opioid antagonist, ADL-8-2698 (Eli Lilly & Co/Adolor Corp), which, in rat brain homogenates, is reported to have the following affinity values for μ-, δ- and κ-opioid receptors (K_i in binding displacement assays): 0.77, 4.4 and 40 nM. However, these *in vitro* data should be considered with caution when considering the ability of a compound to antagonize the constipating effect of narcotics *in vivo*.

Finally, the potential of quaternary opioid antagonists to block the neuromuscular junction or autonomic ganglia must be considered. In the case of MNTX, this effect appears only at high concentrations (160 μM) [434586], [434587].

Pharmacology

According to several early reports in rodents, MNTX reverses the antimotility effect of morphine *in vivo* [434586], [434588]. However, the fact that the drug can be demethylated in these species complicates the interpretation of these studies, especially with regard to the ability of MNTX to concomitantly reverse narcotic-induced analgesia (centrally-

mediated). It is important to ascertain whether the compound can block the antimotility effects of opioids on the gut without interfering with the central actions of morphine. To this end, species that do not significantly N-demethylate (such as the dog) must be used in *in vivo* studies. For a detailed review of these controversial aspects, see Yuan and Foss [423923] and Brown and Goldberg [434586].

The ability of MNTX to antagonize morphine-induced inhibition of smooth muscle contraction *in vitro* was investigated in guinea pig ileum and human small intestine [423935]. In the guinea pig ileum, MNTX (30, 100 and 300 nM) effectively antagonized morphine-induced inhibition (25, 74 and 89% inhibition, respectively). In the human intestine, MNTX at the same concentrations blocked 57, 74 and 92% of morphine-induced inhibition, respectively. These *in vitro* data provide preliminary information supporting the use of the compound in preventing the antimotility effect of morphine.

The lack of central effects of MNTX is demonstrated in studies showing that very large doses of naltrexone methylbromide (1 to 50 mg/kg sc) did not precipitate withdrawal signs in the morphine-tolerant dog, whereas 0.03 mg/kg sc naltrexone did [434588]. Peripheral MNTX (32 mg/kg) also failed to precipitate the withdrawal syndrome in morphine-dependent monkeys [434589]. In addition, MNTX was approximately 10,000-times more potent in reversing morphine-induced catalepsy in rats when given intracerebroventricularly than it was after subcutaneous administration [434590]. Although quaternary naltrexone has lower affinity for opioid receptors, this wide margin probably indicates a difference in the CNS distribution. In guinea pigs, morphine (8 mg/kg ip) completely suppressed cough, and MNTX (0.8 to 2 mg/kg ip) dose-dependently restored the cough response to control levels without affecting analgesia [423934]. By contrast, naltrexone (0.1 mg/kg ip) restored the cough response and blocked analgesia.

A study in dogs demonstrated the efficacy of MNTX (0.2 mg/kg iv or 0.25 mg/kg im) in blocking morphine-induced, peripherally-mediated emesis [423938]. Interestingly, a combination of MNTX (0.25 mg/kg iv, used as a peripheral antiemetic agent) and morphine (1 mg/kg, which is thought to have both a peripheral emetic action and a central antiemetic activity) blocked cisplatin-induced emesis and reduced apomorphine-induced vomiting in the dog [423928].

The gastric effects of MNTX on μ-, κ- and δ-opioid agonist-induced brainstem unitary responses were assessed using an *in vitro* neonatal rat brainstem-gastric preparation [423926]. Single units in the medial subnucleus of the nucleus tractus solitarius (NTS), responding to electrical stimulation of subdiaphragmatic vagal fibers, were recorded. Selective opioid receptor agonists and antagonists were applied only to the gastric compartment of the bath chamber and thus the brainstem functions of the preparation were unaffected by the drugs. The effects of a μ-opioid receptor agonist, DAMGO. and a κ-opioid receptor agonist. U-50488H (Pharmacia & Upjohn Co). were evaluated on 58 tonic units receiving subdiaphragmatic vagal inputs. DAMGO (1.0 μM) and U-50488H (1.0 μM) concentration-dependently inhibited NTS neuronal activity (62 and 49% of the control level.

respectively). MNTX competitively antagonized the DAMGO-induced brainstem neuronal effects and, at an 18.8-fold higher concentration also reversed U-50488H-induced NTS neuronal responses. Naloxone reversed the inhibitory effects of DAMGO and U-50488H at much lower concentrations (3.8% and 0.5%, respectively) compared to MNTX. Only 18% of the NTS neurons evaluated showed inhibitory responses to the δ receptor agonist DPDPE (19% at 10 μM) and this inhibition could not be reversed by MNTX.

MNTX has been used as a pharmacological tool (peripherally restricted opioid antagonist) in several animal studies [17822], [423942], [423943].

Metabolism
Studies of the metabolism of MNTX have demonstrated that there are important species-dependent differences in its N-demethylation to naltrexone [423940]. Rats and mice demethylate the compound over time, as indicated by the exhalation of $^{14}CO_2$ after administration of [^{14}C-methyl]naltrexone, whereas dogs and humans do not significantly demethylate the drug. After intravenous administration of MNTX (2 mg), only one of five patients had detectable amounts of $^{14}CO_2$ in expired breath (peak rate of activity very low; < 0.0001% dose/h at 15 min after treatment) [423940].

Demethylation is important because the resulting tertiary compound has high affinity for opioid receptors and can cross the blood-brain barrier, thus confounding the results of studies investigating the peripheral activity of MNTX. Indeed, the ability of large doses of the drug to antagonize the effects of morphine in rats and mice is currently explained by its significant demethylation in these species [423940]. By contrast, very large doses of naltrexone methylbromide (1 to 50 mg/kg sc) did not precipitate withdrawal signs in the morphine-tolerant dog [434588].

With respect to the other metabolic pathways of MNTX, these have not been studied in detail so far. Significant amounts of the compound (about 50%) are excreted unchanged in the urine after intravenous or subcutaneous administration.

Toxicity
Acute animal toxicity studies (2 weeks) of MNTX have demonstrated a relatively high LD_{50} (> 100 mg/kg ip in rats). In primates, doses of up to 50 mg/kg (iv. sc or im) have been tolerated [423923]. MNTX, at moderate-to-large doses, can block nicotinic ganglionic and cardiac muscarinic receptors; eg, a dose of 5 mg/kg iv can attenuate the cardiovascular effects of 1,1-dimethyl-4-phenylpiperazinium, a ganglion stimulant, the bradycardiac action of methacholine and the reflex bradycardia associated with the administration of phenylephrine [434586]. This effect may explain the orthostatic hypotension observed in a phase I study with an intravenous dose of 0.64 or 1.25 mg/kg iv [282662].

Clinical Development
Phase I
Healthy male volunteers (n = 8) received MNTX in six ascending doses (0.04, 0.08, 0.16, 0.32, 0.64 and 1.25 mg/kg iv) [282662] and were observed for subjective and

hemodynamic changes. Electrocardiogram and laboratory studies were also performed. The dose-limiting, adverse effect of MNTX was transient orthostatic hypotension at 0.64 mg/kg (n = 3) or 1.25 mg/kg (n = 5). Plasma levels of MNTX of > 1400 ng/ml were associated with orthostatic hypotension. There was no release of histamine nor changes in physical examination or laboratory studies during the course of the study. Pharmacokinetic analysis revealed an elimination half-life of 117 min and a clearance of 38.8 l/h after a dose of 0.64 mg/kg. Approximately 50% of the parent compound was recovered in urine after intravenous injection, indicating that renal excretion is a major route of elimination. In conclusion, MNTX was well tolerated at an dose of 0.32 mg/kg iv in healthy humans.

The effectiveness of MNTX in preventing morphine-induced changes in gastrointestinal motility and transit without affecting analgesia was evaluated in 12 healthy volunteers given iv placebo, placebo plus morphine (0.05 mg/kg) or MNTX (0.45 mg/kg) plus morphine (0.05 mg/kg) [423933]. Oro-cecal transit time was assessed by the pulmonary hydrogen measurement technique, analgesia was measured using the cold-pressor test. Morphine significantly increased oro-cecal transit time from 105 to 163 min (p < 0.01). MNTX prevented morphine-induced increase in oro-cecal transit time, but did not affect morphine-induced analgesia. At a higher dose of morphine (0.1 mg/kg), MNTX (0.45 mg/kg) also prevented morphine-induced delay in oro-cecal transit time, with no effect on analgesia. The main pharmacokinetic parameters reported in this study after an iv dose of 0.45 mg/kg were: C_{max} = 3299 ng/ml, elimination $t_{1/2}$ = 131 min, AUC = 677 ng/ml.h, clearance = 52.5 l/h.

In another study [423931], ascending oral doses of MNTX (0.64, 6.4 and 19.2 mg/kg) were first given to 14 healthy volunteers to obtain safety and tolerance data (phase A study). In a single-blind phase B study, these subjects were then given oral placebo and iv placebo, followed by randomized, double-blind, oral placebo and iv morphine (0.05 mg/kg) or oral MNTX (19.2 mg/kg, an established safe dose based on previous administrations of two smaller doses of 0.64 mg/kg and 6.4 mg/kg in phase A) and iv morphine (0.05 mg/kg). Oro-cecal transit time was assessed by the pulmonary hydrogen measurement technique after lactulose ingestion. Morphine significantly increased oro-cecal transit time from 115 to 159 min (p < 0.001), an effect completely prevented by oral MNTX (19.2 mg/kg). These sessions were then followed by single-blind evaluation of descending doses of the drug. Oral MNTX (6.4 mg/kg) significantly attenuated the morphine-induced delay in oro-cecal transit time (p < 0.005 compared with morphine alone), and a dose-dependent response was obtained. There was no correlation between the effects of oral MNTX on the transit time and the drug plasma concentration. This observation suggests the direct preferential luminal effects of oral MNTX. This study also confirmed the low oral bioavailability of the drug. The main pharmacokinetic parameters reported in this study after an oral dose of 19.2 mg/kg were: C_{max} = 166 ng/ml, elimination $t_{1/2}$ = 204 min and AUC = 419 ng/ml.h.

A subsequent trial [423920] evaluated an enteric-coated formulation of MNTX (designed to release the compound in the small bowel and colon) and consisted of two studies: a

pilot study and a controlled study. In the pilot study (n = 3), an oral dose of 6.4 mg/kg of enteric-coated MNTX completely reversed the effects of morphine, producing transit times shorter than baseline levels, whereas the same dose given in an uncoated formulation only partially attenuated the effects of morphine. Subsequently, in the controlled study (n = 9), the transit time increased after iv morphine administration in all subjects, and a lower dose (3.2 mg/kg) of enteric-coated MNTX completely prevented the effect of morphine on transit time. Plasma concentrations after enteric-coated MNTX (6.4 mg/kg and 3.2 mg/kg) were substantially lower compared with those after 6.4 mg/kg of the uncoated formulation. After administration of the uncoated formulation, the unchanged compound detected in the urine from 0 to 6 h was < 0.1 % (compared with < 1% after the oral, uncoated formulation and 50% after intravenous administration in the previous studies), suggesting that the enteric-coated formulation exerts its gut actions more efficiently than the uncoated formulation.

In a recent trial [434591], the efficacy of subcutaneous MNTX in antagonizing morphine-induced delay in oro-cecal transit time was assessed in 12 healthy volunteers. In the first group of subjects (n = 6), morphine (0.05 mg/kg iv) increased the transit time from a baseline level of 85 to 155 min (p < 0.01). After 0.1 mg/kg sc of MNTX plus morphine, the transit time was reduced to 110 min. In the second group of subjects (n = 6), morphine increased the transit time from a baseline level of 98 to 140 min (p < 0.01). After 0.3 mg/kg sc of MNTX plus morphine, the transit time was reduced to 108 min (p < 0.05). In addition, sc MNTX significantly decreased morphine-induced subjective unpleasant symptoms. The main pharmacokinetic parameters reported in this study after a subcutaneous dose of 0.3 mg/kg were: C_{max} = 287 ng/ml, elimination $t_{1/2}$ = 132 min, AUC = 367 ng/ml.h and clearance 55.8 l/h.

The role of peripheral opioid receptor antagonism in modulating opioid-induced delay in gastric emptying was evaluated using MNTX in a randomized, double-blind, crossover, placebo-controlled study [434592]. Eleven healthy volunteers were given either iv placebo, morphine (0.09 mg/kg), or morphine (0.09 mg/kg) plus MNTX (0.3 mg/kg) on three separate occasions before ingesting 500 ml of deionized water. Morphine prolonged gastric emptying as measured by a non-invasive epigastric bioimpedance technique and this effect was reversed by MNTX.

Finally, the efficacy of MNTX, compared with naloxone, in reversing morphine-induced depression of hypoxic ventilatory response was evaluated in ten healthy male volunteers. Naloxone, (5 mg/kg), but not MNTX (0.3 mg/kg), reversed respiratory depression during acute hypoxia [434593]. Thus, MNTX does not appear to penetrate into the brain.

Phase II
In a preliminary phase II study [423924], four subjects with chronic methadone-induced constipation were recruited for a single-blind, placebo-controlled trial for up to 8 days. Placebo was given on the first day; for the remainder of the study MNTX (0.05 to 0.45 mg/kg iv), was administered twice-daily. During the study period, oro-cecal transit time and opioid withdrawal symptoms were recorded, as well as

laxation response based on the frequency and consistency of the stools. Subjects 1 and 2, who were administered MNTX (0.45 mg/kg), showed immediate positive laxation. Subject 2, after a positive laxation response, had severe abdominal cramping but showed no systemic signs of opioid withdrawal. The subject left the study due to the cramping. In subjects 3 and 4, the MNTX dose was reduced to 0.05 to 0.15 mg/kg. The latter two subjects also had an immediate laxation response during and after iv medication without significant side effects. The stool frequency of these four subjects increased from one to two episodes per week before the study to approximately 1.5 stools per day during the treatment period. Oro-cecal transit times of subjects 1, 3 and 4 were reduced from 150, 150 and 150 min after placebo to 90, 60 and 60 min with MNTX, respectively.

In a subsequent double-blind, randomized, placebo-controlled trial [423921], [423945], the efficacy of MNTX (0.01 to 0.365 mg/kg iv daily for 2 days) in treating chronic methadone-induced constipation was evaluated. 22 Subjects enrolled in a methadone maintenance program who had methadone-induced constipation were recruited for this study. The main outcome measures were laxation response, oro-cecal transit time, and central opioid withdrawal symptoms. The 11 subjects in the placebo group showed no laxation response, and all 11 subjects in the intervention group had laxation response after iv MNTX administration (p < 0.001). The oro-cecal transit times at baseline for subjects in the drug and placebo groups averaged 132 and 127 min, respectively. The average change in the drug-treated group was -78 min, significantly greater than the average change in the placebo group (p < 0.001). No opioid withdrawal was observed in any subject, and no significant adverse effects were reported by the subjects during the study.

Pharmacokinetic parameters obtained in this trial for eight subjects receiving the iv dose of 0.08 mg/kg were communicated in a subsequent publication [434591]; C_{max} = 124 ng/ml, elimination $t_{1/2}$ = 129 min, AUC 136 ng/ml.h. Similar findings have subsequently been reported in 12 constipated methadone recipients using oral MNTX at doses of 0.3, 1 and 3 mg/kg (four patients in each group) [423915]. None of the 12 subjects showed laxation response to placebo. Three out of four patients in the 0.3 mg/kg group and all subjects in the other two groups had bowel movements after MNTX. Interestingly, a dose-related reduction of transit time was observed, but nine subjects had undetectable plasma MNTX levels. In the other four subjects, peak plasma levels ranged from 10 to 26 ng/ml. The only reported side effect of the compound was very mild abdominal cramping.

Side Effects and Contraindications
The dose-limiting side effect after intravenous administration is orthostatic hypotension [282662]. Abdominal cramping is another reported side effect [423915], [423924].

Current Opinion
MNTX is an interesting compound for the selective relief of constipation in chronic opioid recipients. If it is conclusively proved safe and effective for this indication, it will fill a gap in the current pharmacological armamentarium. Other potential indications (eg, post-operative ileus or constipation observed in some functional bowel disorders [423923]) that are under scrutiny are at the moment only speculative and should be substantiated by further data, as the role of opioid receptors in these patient populations is still unclear.

Naloxone and ADL-8-2698 are competitors for the same indication. Oral naloxone has been reported to reverse opioid-induced constipation in preliminary studies [434594], [434595]. The rationale for using oral naloxone rests mainly in the limited systemic bioavailability due to first-pass metabolism (about 97%). An enteric-release naloxone formulation is also available [434596]. However, the inherent potential of naloxone to antagonize opioid analgesia and to precipitate withdrawal symptoms [434597] makes this drug a less suitable candidate in comparison with MNTX.

ADL-8-2698 [171235] is a potent, peripherally-restricted, μ-opioid receptor antagonist being developed as a potential treatment for post-operative ileus and opioid bowel dysfunction. Phase III trials in post-operative ileus and opioid-induced bowel dysfunction have been initiated [414887]. Currently, data are insufficient to make a comparative evaluation of these two peripherally-restricted, opioid receptor antagonists.

The results obtained so far with MNTX are promising. However, larger clinical trials with clinically significant endpoints (adequate relief of constipation rather than amelioration of oro-cecal transit measurements) are necessary to support its use as a standard therapeutic measure for opioid-induced constipation. So far, only chronic methadone recipients have been recruited as a proxy group for patients with chronic pain. The optimal route of administration (subcutaneous versus oral) should be established in controlled trials demonstrating whether MNTX can relieve opioid-induced constipation, ensuring, at the same time, maintenance of analgesia in large patient populations. To this end, a range of doses should be tested and the dosing interval should be established. Indeed, the responsiveness to an opioid antagonist might differ between opioid naive and long-term opioid users. Concerning preclinical investigations, dose-response studies carried out in species which do not demethylate MNTX and assessing the ability of the compound to block opioid-induced analgesia and constipation after oral, intravenous and intracerebroventricular administration would allow full evaluation of its peripherally restricted activity.

Licensing
Progenics Pharmaceuticals Inc
In October 2001, Progenics entered into an agreement with UR Labs to obtain exclusive worldwide rights to MNTX. The financial terms of this deal were not disclosed [423791].

UR Labs Inc
MNTX was discovered at the University of Chicago and subsequently licensed by UR Labs [423791].

Development history

Developer	Country	Status	Indication	Date	Reference
Progenics	US	Phase II	Constipation	02-OCT-01	423791
UR Labs Inc	US	Phase II	Constipation	02-OCT-01	423791
University of Chicago	US	Phase II	Constipation	02-OCT-01	423791

Literature classifications

Biology

Study Type	Effect Studied	Experimental Model	Result	Reference
In vitro	Morphine-induced inhibition of contractions.	Guinea pig ileum and human small bowel.	MNTX blocked morphine-induced inhibition of contractions in both preparations.	423935
Ex vivo	μ-, κ- and δ-opioid agonist-induced brainstem unitary responses	Neonatal rat brainstem-gastric preparation.	MNTX reversed the effect of μ- and κ-, but not δ-agonists.	423926
In vivo	Cough suppression and analgesia with morphine.	Guinea pig.	MNTX reversed cough but not analgesia.	423934
In vivo	Emetic effect of morphine.	Dog.	Dose-related antagonism of the emetic effect of morphine.	423938
In vivo	Apomorphine- or cisplatin-induced emesis.	Dog.	MNTX combined with morphine blocks cisplatin-induced emesis and reduces apomorphine-induced emesis.	423928

Metabolism

Study Type	Effect Studied	Experimental Model	Result	Reference
In vivo	N-demethylation of MNTX.	Mice, rats, dogs and humans.	Mice and rats demethylate MNTX; dogs and humans do not significantly demethylate MNTX.	423940
In vivo	Distribution of MNTX into four brain regions.	Rats.	Only 5 to 7% of MNTX found in the brain after ip injection of 10 mg/kg.	434585

Clinical

Effect Studied	Model Used	Result	Reference
Safety and tolerance.	Healthy volunteers (n = 12), phase I study.	MNTX was well tolerated up to 0.32 mg/kg iv. The dose-limiting adverse effect was orthostatic hypotension.	282662
Morphine-induced delay in oro-cecal transit.	Healthy volunteers (n = 14), phase I study.	MNTX (0.45 mg/kg iv) prevented the morphine-induced delay.	423933
Morphine-induced delay in oro-cecal transit.	Healthy volunteers, phase I study.	MNTX (19.2 mg/kg) completely prevented morphine-induced delay.	423931
Morphine-induced delay in oro-cecal transit.	Healthy volunteers, phase I study.	Lower doses of MNTX (3.2 mg/kg po) given in an enteric-coated formulation completely prevented morphine-induced delay.	423920
Morphine-induced delay in oro-cecal transit.	Healthy volunteers, phase I study.	MNTX (0.3 mg/kg sc) reversed the morphine-induced delay.	434591
Subjective, unpleasant effects of intravenous morphine.	Healthy volunteers, phase I study.	Oral MNTX (19.2 mg/kg) decreased the unpleasant effects of morphine.	423929
Laxation response and oro-cecal transit time.	Four chronic methadone recipients with constipation, phase II study.	MNTX (0.05 to 0.45 mg/kg iv) induced laxation and reduced oro-cecal transit time.	423924
Laxation response and oro-cecal transit time.	22 Chronic methadone recipients with constipation, phase II study.	MNTX (0.09 to 0.10 mg/kg) induced laxation and reduced oro-cecal transit time.	423921 423945
Laxation response and oro-cecal transit time.	12 Chronic methadone recipients with constipation, phase II study.	MNTX (0.3 to 3 mg/kg po) induced laxation and reduced oro-cecal transit time.	423915

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Effect of μ-opioids morphine and buprenorphine on the development of adjuvant arthritis in rats

J. S. Walker[1], A. K. Chandler[1,2], J. L. Wilson[1], W. Binder[1] and R. O. Day[1,2]

[1]School of Physiology & Pharmacology, University of New South Wales, Sydney 2052, Australia
[2]Department of Clinical Pharmacology, University of New South Wales, Sydney 2052, Australia

Abstract. *Objective and Design:* On the basis that endogenous opioids play a role in the physiological response to inflammation, this study tests the anti-arthritic effects of a μ-opioid agonist, morphine and the partial μ-agonist, buprenorphine.
Material: Male Lewis rats were used.
Treatment: Rats were innoculated subcutaneously with 0.05 ml of Freund's complete adjuvant (5 mg/ml) into the right hind paw to produce adjuvant arthritis. Morphine (either 10 to 60 mg/kg/day s.c. bolus or 60 mg/kg/day s.c. infusion) and buprenorphine (0.65 ± 0.06 mg/kg/day, orally), respectively, were administered for 3 days during the primary inflammatory phase of adjuvant arthritis.
Methods: The progression of adjuvant arthritis was monitored every three days by body weight change and hind limb oedema (ipsilateral and contralateral). On day 21 the animals were sacrificed and histology and radiography of the contralateral limb were performed. In rats receiving Freund's adjuvant and no drug treatment, the incidence of arthritis was 89%. Effect was expressed as the pooled severity index (PSI) derived from the arithmetic average of the volume, histology and radiography scores in the contralateral hind limb.
Results: Buprenorphine had no effect on experimental arthritis (PSI control vs treated: 242 ± 28 vs 253 ± 28%). In contrast, morphine by subcutaneous injection twice daily (10 to 60 mg/kg/day) but not by subcutaneous infusion (60 mg/kg/day) was found to attenuate the progression of adjuvant arthritis in a dose-dependent manner. This indicates that the anti-arthritic effects of morphine are opioid receptor mediated (ED_{50}, 58 ± 9 mg/kg) and suggests that the local concentration reached effective levels only after subcutaneous injection. It is also possible that the high doses of morphine were anti-inflammatory through effects at the kappa receptor. However, these high doses of morphine produced death in one third of the rats, the calculated lethal dose (LD_{50}, 63 ± 2 mg/kg) being close to the effective dose.

Conclusion: Anti-arthritic effects of morphine are opioid receptor mediated but morphine use for this indication is restricted by its adverse effects.

Key words: Opioids – Morphine – Buprenorphine – Adjuvant arthritis

Introduction

Rheumatoid arthritis is characterised by inflammation of the synovial lining, massive accumulation of T lymphocytes and mononuclear phagocytes, proliferation of synovial cells and erosion of bone and cartilage [1]. Conventional therapies for inflammatory arthritis have not included the use of opioid drugs largely because of lack of proven efficacy in humans for this condition and their addictive properties. However, opioids are known to affect both inflammatory states [2, 3] and immune processes underlying arthritic disease [4, 5]. We and others have shown that opioid drugs are able to attenuate the severity of adjuvant arthritis in rats [6–9].

There are three families of endogenous opioids and three receptor classes [10–12]. Endogenous and exogenous opioids act upon mu (μ), delta (δ) and kappa (κ) receptor classes to evoke a broad spectrum of effects (for review see [10, 13]). The importance of opioid receptor subtypes with respect to inflammation remains to be determined. Morphine has been reported to have both stimulatory and inhibitory effects on the immune system [14]. Similarly, it can both facilitate [15] and inhibit the progression of adjuvant arthritis [6]. For example, infusion of morphine intracerebroventricularly over 2 days (0.9 mg/kg/day), was found to result in less severe arthritis as judged by radiological criteria [6]. It has been suggested, therefore, that morphine may interfere with descending neuronal controls and that interruption of the innervation of the joint may be of benefit [6]. In contrast, another laboratory has found that low systemic doses of

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Correspondence to: J. S. Walker

morphine (8.5 mg/kg/day) exacerbates the disease [15]. In addition, the κ-opioid agonists, (±) U-50,488H and trifluadom have been found to inhibit carrageenan-induced paw swelling in the rat [16] and we have found that the κ-opioid U-50,488H attenuates experimental arthritis in a dose-dependent, antagonist reversible and stereoselective manner indicating that it exerts its effects via opioid receptors [8, 9].

Against this background of conflicting data with the μ-agonist morphine, the primary aim of the present study was to investigate the effects of the prototype μ-opioid receptor agonist morphine in experimental inflammatory arthritis. Because the partial μ-agonist buprenorphine is now commonly used as a post-operative analgesic in rats [17] and because of a brief report claiming it exacerbates inflammation [18] we also evaluated buprenorphine in experimental arthritis. A secondary aim of the study, based on the hypothesis that, a constant delivery of morphine would provide better control of the disease, was to compare the anti-arthritic effects of different dosing regimens of morphine, i.e. intermittent versus continuous administration.

Materials and methods

Male Lewis rats (Animal Resources Centre, Perth, Australia), weighing approximately 200 g at the start of the study, were used. These animals were housed in groups of 10 in large cages in a climate controlled room with lights on from 6 am to 6 pm for one week before and throughout the study. All experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales and The Garvan Institute, St. Vincent's Hospital, Sydney, Australia.

To produce adjuvant arthritis, rats were anaesthetised with methoxyflurane and injected with 50 μl of complete Freund's adjuvant (5 mg/ml suspension in paraffin oil and mannide mono-oleate, Difco Laboratories, Detroit, MI) subcutaneously into the right hind paw on day 0. Control rats received similar injections of incomplete Freund's adjuvant (paraffin oil and mannide mono-oleate, Sigma Chemicals, USA).

In the first experiments we followed the protocol of Levine and co-workers who evaluated the effects of morphine by intracerebro-ventricular injection over 29 days [6]. Subsequently, careful observations in our laboratory showed that the adjuvant arthritis peaks between days 18 and 21 as judged by plethysmometry and histology of the contralateral paw, although the radiographic changes persist until at least 30 days post-induction (unpublished data). On ethical grounds, we therefore carried out subsequent experiments for 21 days. The progress of the disease was monitored by measurement of both hind limb volumes (ipsilateral and contralateral) by plethysmometry (Ugo Basile, Comerio, Italy) and body weight approximately every 3 days for a total of 29 days (buprenorphine) and subsequently for 21 days (morphine) when all arthritic parameters were at a peak.

Serum sulphydryl (SH) concentrations were monitored to determine their usefulness as a biochemical index of arthritis severity and as a predictor of response to therapy. Serum was reacted with Ellman's reagent (0.05 mM- 5,5'-dithio-bis-(2-nitrobenzoic acid in 0.1 M phosphate buffer, Sigma) and the intensity of the colour reaction determined at an absorbance maximum at 412 nm [19].

To assess joint damage, control and treated rats were sacrificed (pentobarbitone, 60 mg i.p.) on Day 21 (morphine dose-response experiments) or Day 29 (buprenorphine) post-adjuvant and the contralateral paw was removed. Radiology and histology were then performed on the contralateral paw (left leg) to assess joint damage.

all radiographs were performed using a dedicated mammography machine (General Electric 600T) at a fine focus of 0.3, an aluminium filter and Mammoray MR5 film (Agfa). The exposure was 27 kV and 4 mA.s for bony detail and 24 kV and 4 mA.s for assessment of soft tissue swelling respectively. The specimens were radiographed at a focus to film distance of 65 cm and viewed at a magnification of 1.85. The following parameters were evaluated without knowledge of the treatment [20]:

1. Soft tissue swelling: increased width in the soft tissue shadows and alterations in the normal configuration of soft tissue shadows indicating oedema, effusion, and possibly synovial thickening.
2. Erosion: destruction of bone architecture seen as increased radiolucency developing at the site of erosions.
3. Osteoporosis: decreased density of the bone recognised as increased radiolucency relative to uninvolved adjacent bone, and thinning of the cortex, particularly in the juxta-articular regions.
4. Joint space loss: narrowing of the joint spaces owing to loss of cartilage.
5. Joint damage: destruction of normal architecture and config-uration of the joint.

A subjective rating scale was used to grade each parameter with 0 indicating normal, 1 indicating mildly, 2 moderately and 3 severely affected to give a maximum possible score of 15 [8].

Immediately following radiography the specimens were pre-served in neutral buffered 10% formalin for 7 to 10 days. After fixation, the specimens were decalcified in 30% formic acid for 3 to 5 days. Longitudinal specimens were prepared such that the dorsoventral faces of the tarsal, metatarsal and phalangeal joints were presented. The specimens were then dehydrated, infiltrated with and embedded in paraffin wax, cut into 7 μm-thick sections and stained with haematoxylin-eosin [20].

Each specimen was evaluated, by an observer who had no knowledge of treatment, for character of pathologic change noting the number of joints involved and the level of damage at each joint including:

1. proliferation of tissues accompanying the synovitis taking into consideration the type of cells (neutrophils, macrophages, lymphocytes, plasma) present.
2. presence of pannus.
3. destruction of articular cartilage.
4. proliferation of periarticular periosteal bone.

A subjective rating score for each parameter from 0 to 8, based on the method described by Ackermann and co-workers, was adopted, the maximum possible score being 32 [20].

Drugs

The opioid drugs used were morphine sulfate and buprenorphine (Temgesic®). They were administered for 3 days only during the primary inflammatory phase of adjuvant arthritis (days 0 to 2).

Buprenorphine. For comparison with previous studies [18], bupre-norphine was administered via the drinking water (supplemented with 5% dextrose) for 3 days (0.005 mg/ml) (Table 1). Preliminary experiments demonstrated that higher doses produced sedation in the rats. Control rats (n = 12) received adjuvant vehicle alone, and twenty-three rats treated with complete Freund's adjuvant received either dextrose water (n = 11) or buprenorphine (n = 12). Water intake was monitored daily for each treatment group to calculate the dose of buprenorphine delivered. On average each rat would have received buprenorphine 0.13 ± 0.04 mg/day/rat (mean ± se: 0.65 ± 0.06 mg/kg/day). Based on an oral availability of 10 to 20% [21] this dose exceeds the usual clinical dose of 0.05 mg/kg s.c [22]. *Morphine.* Two separate experiments were carried out to deter-mine the anti-arthritic effects of morphine (Table 1). Firstly, the

Table 1. Pooled Severity Index[a].

Group & dose (mg/kg/day)	Buprenorphine (orally)	Morphine (s.c. bolus)	Morphine (s.c. infusion)
Nonarthritic Arthritic	13 ± 39 (12)		3.4 ± 17 (7)
0	242 ± 28 (11)	309 ± 17 (21)	144 ± 16 (12)
0.75	253 ± 28 (12)		
10		268 ± 40 (5)	
20		263 ± 36 (5)	
40		228 ± 36 (10)	
60		152 ± 26 (15)	132 ± 16 (12)

[a] Results are expressed as mean ± SE, n is given in parentheses following the number.

anti-arthritic effects of morphine were examined using subcutaneous (s.c.) bolus injections into a skin fold in the back of the neck, twice daily, for the first 3 days over the dose range: 0 mg/kg/day (n = 21); 10 mg/kg/day (n = 5); 20 mg/kd/day (n = 5); 40 mg/kg/day (n = 10) and 60 mg/kg/day (n = 15), while nonarthritic controls received morphine by s.c. bolus injection (60 mg/kg/day, n = 7).

Morphine sulphate was also administered via ALZET osmotic minipumps implanted subcutaneously for 3 days to deliver a continuous infusion of morphine at a rate of 60 mg/kg per day. Morphine (66.4 mg morphine sulphate) was dissolved in 33% polyethylene glycol 400 to ensure that it remained in solution inside the pumps. Arthritic rats received either morphine (n = 12) or saline (n = 12), while nonarthritic controls received morphine by infusion (60 mg/kg/day, n = 7).

Drug analysis

To confirm the effectiveness of the osmotic pumps, blood was obtained via the caudal artery in anaesthetised rats on day 3 immediately prior to pump removal and serum morphine concentrations were assayed by radioimmunoassay [23]. We have previously found that this assay is specific for morphine and compares well with chromatographic techniques [23].

Data treatment

Data are presented as mean ± SEM. Volume measurements were normalised as % change from day 0. Statistical analyses were performed by repeated measures factorial ANOVA after assessing the data for normality using Shapiro and Wilk's test [24]. Such analyses were used to calculate the average change from day 0 with respect to time. Ordinal data such as radiography and histology scores were normally distributed. Our experimental model of inflammatory arthritis is a multifaceted disease and we have therefore sought to make our severity index comprehensive [9]. The three indices of arthritic damage – the time-averaged contralateral hind limb volume, radiography and histology – were thus expressed as a percentage of vehicle treated arthritic rats (control) and totalled to obtain a 'pooled severity index' (PSI). Animals were judged to be arthritic if this score increased 2 SD above that of the mean of the non-arthritic control group. The PSI was subjected to parametric statistical analysis (one-way ANOVA). Multiple comparisons were performed by the Newman-Keuls test [20]. $P < 0.05$ was deemed statistically significant. All analyses were performed with the Number Cruncher Statistical System V 6.06 (NCSS, Utah, USA).

For the analysis of morphine dose-response data, effect was also expressed as a PSI. The PSI in opioid-treated rats was then expressed as a percentage of vehicle treated arthritic rats to obtain an index of disease attenuation. Dose-response relationships for morphine were analysed by fitting the percent attenuation data to the nonlinear form of the Hill equation (sigmoid E_{max}, model) using nonlinear regression procedures (PCNONLIN [25]). E_{max} was fixed at 100% and the following parameters were obtained: the dose at half maximal effect, ED_{50}, and S, an estimate of the steepness of the curve.

The LD_{50} for morphine (the dose at which 50% of the animals did not survive) was obtained by probit analysis according to Litchfield and Wilcoxon [26] using the software program PROBITAN (K. L. McGilliard, Zoology Department, Eastern Illinois University, Charleston, IL, USA).

Results

In animals receiving complete Freund's adjuvant and no drug treatment the incidence of arthritis was 89% (40 out of 45 rats) as judged by an increase in the pooled severity
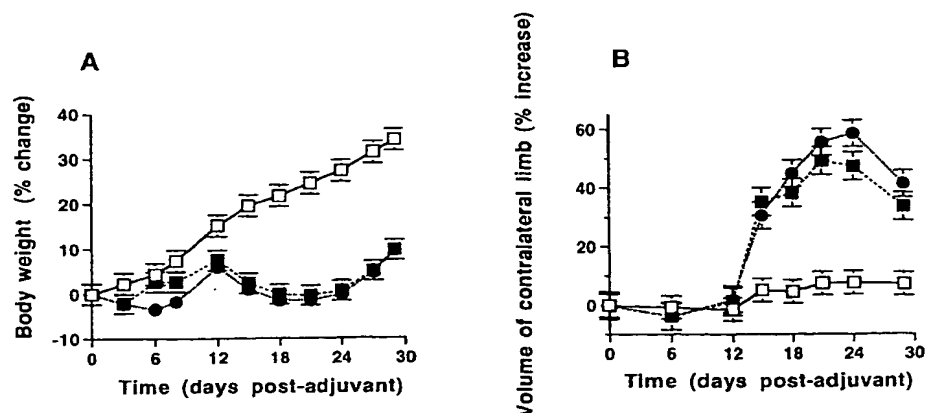

Fig. 1. Mean ±SE in (A) body weight (% change from day 0) and (B) contralateral (left) paw volume (% change relative to day 0) as a function of time in non-arthritic rats (control, □, n = 12) and arthritic rats treated with either vehicle (n = 11, ■) or buprenorphine (n = 12, ●) in the drinking water to deliver 0.65 mg/kg/day for 3 days (0 to 2).
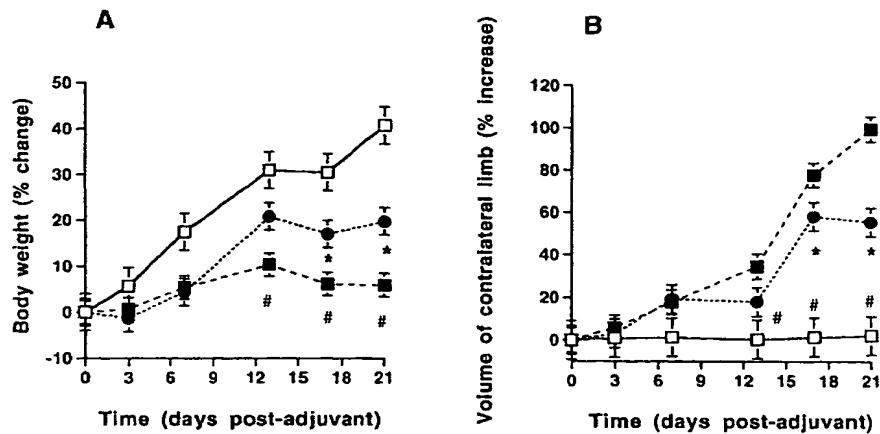
A

B

Fig. 2. Mean ± SEM in (A) body weight (% change from day 0) and (B) contralateral (left) paw volume (% change relative to day 0) as a function of time in non-arthritic rats, n = 7 (control, □) and arthritic rats treated with either vehicle (no opioid, n = 12, ■) or morphine (n = 11, ●) by subcutaneous bolus injections twice daily to deliver 60 mg/kg/day for 3 days (0 to 2). *#p < 0.05 compared to vehicle-treated arthritic rats.

index (PSI) in the contralateral hind limb. Control animals injected with adjuvant vehicle (incomplete Freund's adjuvant) did not demonstrate any signs of arthritis. Rats with arthritis were able to maintain their body weight albeit with a slower growth rate (Figs. 1A–3A) with signs of recovery by Day 30 in the buprenorphine experiment (Fig. 1A). The disease progressed to polyarthritis as characterised by a peak in the volume of the contralateral limb between days 18 and 21 post-adjuvant (Fig. 1B and 3B). Compared to nonarthritic rats, serum sulphydryl concentrations were decreased in arthritic rats (mean ± se (% of control); non arthritic vs arthritic: 105 ± 3 vs 33 ± 4%, n = 59, p < 0.05).

Buprenorphine

Buprenorphine (0.65 mg/kg/day) had no effect on the progression of experimental arthritis as judged by paw swelling in either the ipsilateral (data not shown) or contralateral limb (Fig. 1B). Buprenorphine treatment also had no effect on serum sulphydryl concentrations in arthritic rats (vehicle *cf* buprenorphine: % change from day 0 to day 29: 25 ± 2 *cf* 24 ± 4%). The PSI was not different in arthritic rats receiving either vehicle or buprenorphine (242 ± 28 *cf* 253 ± 28, p > 0.05, Table 1). Preliminary experiments demonstrated that sedation occurred at doses above 0.65 mg/kg/day (unpublished data), thus higher doses of buprenorphine were not investigated.

Morphine

Morphine had no effect on the ipsilateral paw volume in arthritic rats over the duration of the study (mean ± se% increase from day 0 to day 21: morphine *cf* no morphine: 80 ± 3 *cf* 83 ± 3%, n = 12 per group, p > 0.05). Further, morphine treatment had no effect on serum sulphydryl

concentrations in arthritic rats (vehicle cf morphine: % change from day 0 to day 21 : 33 ± 4 *cf* 28 ± 4%, n = 12, p > 0.05).

Dose-response relationships. The dose-response relationship for morphine was determined by twice daily subcutaneous (s.c.) injections, for 3 days during the primary inflammatory phase (days 0 to 2) over the dose range 10 to 60 mg/kg/day s.c. The lowest dose, 10 mg/kg/day, produced no mortality. Doses of morphine above 10 mg/kg/day s.c. produced respiratory depression following the first and second doses resulting in a total of 12 deaths (n = 35); one after 20 mg/kg/day, five after 40 mg/kg/day and six after 60 mg/kg/day, s.c. The LD_{50} was calculated to be 64 ± 2 mg/kg (n = 35).

Mean ± SE% change in both body weight and contralateral paw volume as a function of time following the 60 mg/kg/day by subcutaneous bolus is presented in Figures 2A and 2B. Arthritic damage, as judged by the PSI was decreased in a dose dependent manner by morphine over the range 10 to 60 mg/kg/day (Table 1, Fig. 4). Analysis of dose-response data revealed an ED_{50} of 58 ± 2 mg/kg/day with S (steepness parameter) 1.7 and r = 0.95 (p < 0.05, n = 35) (Fig. 4).

Subcutaneous infusion of morphine

The rationale was to determine if a constant delivery of morphine could provide better control of the disease without the mortality associated with intermittent subcutaneous injections. Thus, a dose similar to the estimated ED_{50}, 60 mg/kg/day was delivered for 3 days via mini osmotic pumps. Unlike subcutaneous bolus injections, this route produced no mortality. Serum morphine concentrations after subcutaneous infusion were found to be 75 ± 12 ng/ml (range 61 to 108 ng/ml; n = 12). Constant delivery of morphine had no significant effect on body weight change and contralateral hind limb
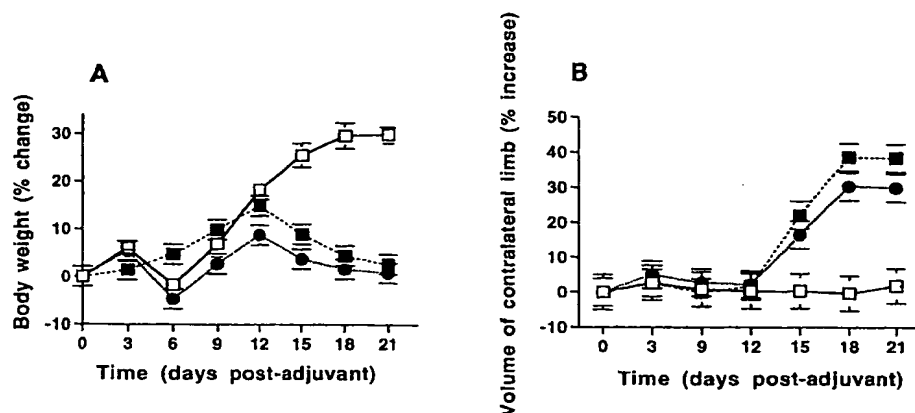
Fig. 3. Mean ± SEM in (A) body weight (% change from day 0) and (B) contralateral (left) paw volume (% change relative to day 0) as a function of time in non-arthritic rats, (n = 7, control, □) and arthritic rats treated with either vehicle (no opioid, n = 12, ■), or morphine, (n = 12, ●) by subcutaneous infusion via osmotic pumps to deliver 60 mg/kg/day for 3 days (0 to 2).

volume measurements (Fig. 3A and 3B). Similarly, sulphydryl concentrations were not altered by morphine treatment [saline controls *cf* morphine (n = 12 per group); % change from day 0 to day 21; 34 ± 7 cf 25 ± 7, p > 0.05]. In view of the lack of effect of morphine by subcutaneous infusion on contra-lateral hind limb volume, radiography and histology measurements were not carried out here because, in our experience, differences in these latter parameters do not occur if there are no plethysmometry changes [8].
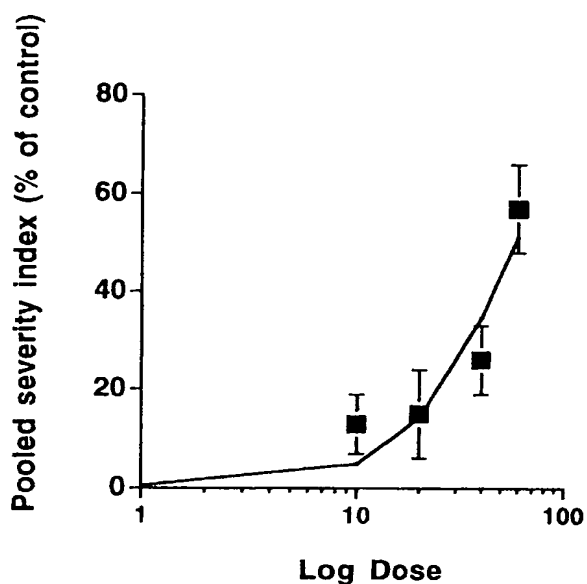

Fig. 4. Effect of morphine on arthritis severity after 10 to 60 mg/kg/ day by subcutaneous bolus injection twice daily for 3 days (0 to 2) as determined by nonlinear regression to an Emax form of the Hill equation. The ED50 and slope function were 58 ± 2 and 1.7 ± 0.5, respectively (n = 4 to 9 animals per dose).

Discussion

Adjuvant arthritis in male Lewis rats produces a reliable animal model of experimental arthritis. The rats appear well groomed and on average are able to sustain their initial body weight over 21 to 30 days. Further, injection of adjuvant into a hind paw allows one to monitor the systemic progression of the disease as judged by an increase in swelling of the contralateral hindpaws. Treatment with either buprenorphine or morphine by any route used did not affect the volume of the ipsilateral paws or serum sulphydryl concentrations. These findings confirm our earlier work with this model [27]. Thus, although serum sulphydryl concentrations may be a useful biochemical index to confirm the presence of arthritic disease in this model of adjuvant-induced arthritis, they were of little value in detecting treatment effects in this study.

Buprenorphine is a mixed agonist and antagonist, acting both as a partial μ-agonist [28] and an antagonist at κ-receptors [29]. In the present study, buprenorphine (0.65 mg/kg, orally) did not alter experimental arthritis although there was a nonsignificant trend towards exacerbation. The latter result supports that of other investigators who were able to demonstrate that this opioid (0.4 mg/kg, orally) exacerbates the inflammatory response, as judged by paw swelling and lameness [18]. It may be important that in this latter study, buprenorphine was not administered until the inflammation had been established whereas in the present study it was administered during disease onset. Others have found that the prototype antagonist, naloxone, which acts at all opioid receptor subtypes, also exacerbates inflammation [30]. In contrast, high doses of naloxone (72 mg/kg) but not low doses (3.8 mg/kg/day) were found to prevent the contralateral transfer of experimental arthritis [31]; it has been suggested that this in fact could be an agonist action [32, 33]. Intermittent subcutaneous administration of buprenorphine to produce higher peaks may have been associated with efficacy. However, it is unlikely that

higher doses of buprenorphine would be beneficial in adjuvant arthritis because it acts as an antagonist at kappa receptors [29] and we have found previously that κ-opioid antagonists are devoid of anti-arthritic effects [8].

Morphine (a μ-agonist), administered by subcutaneous bolus twice daily exhibited anti-arthritic effects in rat adjuvant arthritis but morphine by subcutaneous infusion has no significant anti-arthritic effect. Further, the effective dose for 50% response (ED_{50}, ~60 mg/kg/day) was found to be close to the lethal dose (LD_{50} ~ 64 mg/kg/day). Interestingly, the serum morphine concentrations after subcutaneous infusion, i.e. steady-state (~75 ng/ml) were comparable to effective analgesic concentrations seen in young humans (4 to 65 ng/ml, [34]. Our data which indicates that intermittent but not continuous administration of morphine attenuates arthritis suggests that the effect is dependent upon the peak concentration of morphine reached at the site of action. Based on published data it can be predicted that serum concentrations of morphine after a 60 mg/kg/day s.c. injection would be well in excess of 1000 ng/ml [35]. Similar effects have been noted for the degree of analgesia attained after administration of analgesic drugs (for review see [36]). Thus a higher dose infusion of morphine would probably be effective. Alternatively, continuous administration of morphine via osmotic pumps may result in tolerance and thereby account for the lack of anti-arthritic effect.

The effect of subcutaneous bolus doses of morphine in this arthritic model was dose-dependent suggesting that morphine mediates its anti-arthritic effects via interactions with opioid receptors. We previously found that administration of the κ-opioid agonist ($\pm$)U-50,488H attenuated the severity of polyarthritis in rats in a dose-dependent and antagonist reversible manner [8, 9], and others have found ($\pm$)U-50,488H inhibited inflammation in monoarthritis models [16]. In the present study, it is also possible that the high doses of morphine were anti-inflammatory due to effects at the kappa receptor [37].

Morphine (180 µg/day for 2 days) administered via intracerebroventricular infusion has been found previously to attenuate the radiological damage in a polyarthritic rat model [6]. From this result, the authors concluded that opioid drugs can block neurogenic inflammation via blockade of descending neural circuits [6]. Morphine by this very high dose could also have exerted its effects via the systemic route. Alternatively, opioids may be exerting their anti-arthritic effects via the hypothalamus. Endogenous and exogenous opioids are also known to affect the hypothalamicpituitary-adrenocortical axis (HPA) with contrasting effects depending on the administration mode (acute *cf* chronic). For example, acute administration of morphine, to activate μ-opioid receptors, increases the release of neurohormones, like corticosterone, from the HPA axis [38]. In contrast, chronic treatment with μ-opioid agonists results in a decreased responsiveness of the HPA axis to stress in rats (for review see [39]). Morphine administered subcutaneously by osmotic pumps to deliver 8.5 mg/kg/day for 18 days exacerbated experimental arthritis [15]. It is possible, therefore, that continued use results in an

inability of the animals to produce adreno-corticotrophic hormone in response to stress via an effect on the hypothalamopituitary-adrenal axis [40]. This would result in increased susceptibility to adjuvant arthritis [41].

Data on the mechanisms of anti-inflammatory/anti-arthritic effects of morphine are not only equivocal but contradictory. Further experiments are required to elucidate the exact mechanism(s) involved, in light of the complex interrelationships between the immune, neural and neuroendocrine systems and because morphine has dose-dependent stimulatory and inhibitory effects on all three systems [14, 42, 43].

In conclusion, μ-opioid agonists like morphine may be effective anti-arthritic agents if administered as intermittent injections during the early phase of inflammatory arthritis. However, the usefulness of morphine itself is limited by its narrow therapeutic index. In contrast, mixed opioid agonists and antagonists, like buprenorphine, are devoid of anti-inflammatory effects. Opioid receptors have now been located on peripheral nerve endings [44] and opioid peptides have been found within immune cells, such as macrophages and T-lymphocytes, at sites of inflammation [45, 46] thus a strategy to improve the adverse side effect profile of opioids, like morphine, is to restrict the access of such compounds to the central nervous system. Opioid drugs that are unable to cross the blood brain barrier could open up new avenues of treatment for chronic inflammatory disorders.

Note added in proof. Since this paper went to press, Hall et al. (Inflamm Res 1996;45:299–302) have reported that, in the rat adjuvant arthritis model, buprenorphine exacerbates inflammation as measured by paw volume and radiography.

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The Anti-inflammatory Effects of Opioids: Their Possible Relevance to the Pathophysiology and Treatment of Rheumatoid Arthritis

Judith S Walker[1], Jodie L Wilson, Waltraud Binder, Caroline Scott & John J Carmody

Address
[1]Corresponding Author
Dr Judith S Walker
School of Physiology & Pharmacology
University of New South Wales
Sydney 2052
Australia
Fax +61 (02) 9385-1059
Tel +61 (02) 9385-3810
E-mail: Judy.Walker@unsw.edu.au

Introduction

Inflammation is involved in many pathological conditions of which one of the most common and severe is rheumatoid arthritis (RA). This is a chronic inflammatory polyarthritis which has a world-wide distribution, affecting approximately 1-2% of the population particularly women. Its cause is a puzzle to modern medicine. The prevailing view is that the disease involves a genetic predisposition together with antigen activation of T-lymphocytes which then infiltrate the synovial membrane [1]. The result is vascular and synovial cell proliferation with resorption of bone and cartilage. These events involve excessive release of inflammatory mediators, such as cytokines (from cells including macrophages and mast cells) under both humoral and neural influences [2, 3]. Cytokines stimulate bone resorption, T-cell activation and can induce expression of adhesion molecules (eg intercellular adhesion molecule-1, ICAM-1) that enhance adherence of neutrophils, monocytes and lymphocytes to the endothelium [4]. Rheumatoid arthritis is a disease that is characterized by joint pain, swelling and stiffness accompanied by structural damage and deformity of multiple joints which inevitably lead to disability and premature death [5]. In fact, 70% of patients present with radiographic erosions and joint space narrowing during the first three years of the disease.

It is not sufficiently appreciated that patients with RA may have a five-year mortality similar to patients with cardiovascular or neoplastic disease [6]. Prevention of disability and death is the ultimate goal of treatment. As is the case for many other chronic diseases, however, no cure is yet available. Instead, current treatment is aimed at relieving symptoms and improving functional performance. There is now a growing recognition that patients with RA require treatment early in the disease before the development of erosions and deformity, and this argues strongly for the re-evaluation and improvement of strategies for RA therapy.

Traditionally, RA has been treated using a pyramid approach [7]: firstly, with non-steroidal anti-inflammatory drugs (e.g. aspirin) which are the mainstay of therapy,

then as the disease worsens, second line agents or disease modifying antirheumatic drugs are introduced (such as gold), followed (as the third level of the pyramid) by hydroxychloroquine, and lastly immunosuppressive drugs. These current therapies produce toxicity in at least 30% of patients but none of these potentially toxic agents is capable of controlling the long term morbidity and mortality associated with this disease. The efficacy of this conservative approach has been challenged in recent years (for review see [6]). More successful treatment, especially early in the disease, is clearly required: reducing the delay from disease onset and diagnosis to initiation of effective therapy is of paramount importance. While biological agents including antibodies to cytokines (eg. tumor necrosis factor-α, TNF-α) and ICAM-1 and gene therapy (for review see [8]) have promise, work by Walker et al [9, 10] has re-focused attention on opiates which, though widely used as analgesics, have not previously been seriously considered as anti-inflammatory agents, much less as a therapy for RA. This review will focus on the potential use and mechanisms of action of opioids as anti-inflammatory agents, with particular emphasis on RA.

Opioids: general considerations

It is generally believed that opiate drugs (opium derivatives) act at receptors for endogenously-occurring opioid substances. There are three families of these endogenous opioids and three receptor classes but, as yet, no direct correspondence between them has been established (for review see [11]). The agonists, coded by separate genes, are: (a) β-endorphin (derived from pro-opiomelanocortin), (b) met- and leu-enkephalin (from pro-enkephalin) and (c) the dynorphins (arising from pro-dynorphin) [12]. The three receptor classes, named mu (μ), delta (δ) and kappa (κ), have been cloned and each mediates different physiological and biochemical processes; these cloned receptors have a high degree of homology and the seven-pass transmembrane quaternary structure which is typical of the superfamily of G-protein linked receptors [13-18]. By means of immunohistochemical techniques, with specific antibodies raised against the cloned receptors, the distribution of opioid receptors within the rat CNS has been thoroughly studied [19].

Endogenous and exogenous opioids act upon these receptors to evoke a broad spectrum of effects, not just analgesia (for review see [11, 20]). The clinical use of opioids, therefore, is restricted by the plethora of unpleasant side-effects which are associated with these drugs, notably when used against chronic diseases such as arthritis. In this regard, κ-opioid agonists possess some advantages over μ-agonists: they are devoid of such side effects as dependence liability, constipation and respiratory depression [21, 22]. For these reasons drug companies have pursued the possibility of κ-agonists as analgesic agents. However, they possess a spectrum of

other side-effects including CNS disturbances and dysphoria [23, 24]. These disadvantages need to be overcome before they can be employed in chronic inflammatory disease.

Anti-inflammatory effects of opioids

A great deal is known about the analgesic effects of opioids [20]. Barber and Gottschlich [25] have comprehensively reviewed the actions of opioids on the hyperalgesic aspects of inflammation. Apart from the research being undertaken by Walker's group there have been relatively few studies of their peripheral anti-inflammatory effects, so a brief overview of these effects is presented here. Both μ (morphine) and κ-opioid (U50488H, trifluadom) agonists can inhibit carrageenan induced paw swelling in the rat by up to 40% [26-30]; moreover, this action of morphine is dose-dependent and partially antagonized by opioid antagonists [29, 30]. Morphine also significantly reduces the neurogenic edema response to saphenous nerve stimulation, an effect which is antagonized by naloxone [31], as well as the plasma extravasation induced in the knee-joint by capsaicin (which is believed to be dependent upon both primary afferents and post-ganglionic sympathetic fibers) [32]. On the other hand, a selective μ-agonist (Try-d-Ala-Gly-NMe-Phe-Gly-ol) has been found to be without effect on bradykinin-induced extravasation (which is principally dependent on sympathetic post-ganglionic neurons) whereas this extravasation is attenuated by specific δ- and κ-agonists [32]. A similar pattern is observed when opioids are used with the inflammation induced in rat skin by reactive oxygen species [33].

Opiates also influence the inflammatory processes of rheumatoid arthritis; this property together with their analgesic actions, strongly suggests a potential therapeutic role in this disease. In 1985, Levine and colleagues [34] showed that central (ICV) administration of high doses of morphine attenuated the development of experimental arthritis, and in 1996 Walker and co-workers [35] showed that morphine (albeit at high doses) attenuates the inflammation and progress of this disease when administered systemically. Such high doses would preclude clinical use of opioids in arthritis so more specific attention was given to κ-agonists, partly because of the analgesic action of dynorphins and partly because their agonist-receptor relations are better understood. Walker's group found that the specific κ-agonist, U50488H, attenuated adjuvant arthritis in a dose-dependent, stereoselective, antagonist-reversible manner (using the comprehensive criteria of paw swelling, radiography and histology) [10].

However, there are still some puzzling results. Morphine, in low doses has been found to facilitate the progression of adjuvant arthritis [37] and Hall et al [38] found that the mixed μ-agonist/κ-antagonist, buprenorphine, also exacerbated adjuvant arthritis. Walker's group found, though, that neither buprenorphine [35] nor the pure κ-antagonist MR-2266 [9] had any effect in this disease. Paradoxically, the prototypical μ-opioid antagonist, naloxone, attenuates experimental arthritis at high, but not low doses [38], an effect which has been suggested to be due to an agonist action [39, 40], though naloxone acts on all receptor classes [20].

The site of action of the anti-inflammatory effects of opioids

Historically, opioids have been thought to produce their antinociceptive effects via actions in the central nervous system. More than a century ago, however, Wood demonstrated that morphine elicited analgesic effects when applied locally to "painful areas" in the periphery [41]. This finding has now been confirmed in clinical studies in which intra-articular morphine produced pain relief following knee arthroscopy, without systemic effects [42]. Similarly, a number of studies have reported that intra-articular administration of opioids can produce pronounced analgesic effects, by interacting with peripheral opioid receptors in inflamed tissue (for review see [43]). To date, κ-opioids have not yet been used clinically in this way for lack of an appropriate drug.

The relative importance of different opioid receptor subtypes involved in inflammation has yet to be elucidated, however, several lines of evidence suggest that primary afferent neurones are equipped with opioid receptors: i) opioid receptors have been located on afferent terminals in joints [44-49]; ii) opioid agonists acting at peripheral receptors are analgesic, eg intraplantar fentanyl reverses rat paw hyperalgesia in a naloxone-sensitive manner [50] and, as mentioned above, local administration relieves post-arthroscopy pain in patients [42]; iii) close arterial injection of κ-agonists and, to a lesser extent, μ-agonists into the joint inhibits activity in afferent fibers [44]; and iv) the release of the neuropeptide, substance P (SP), from primary afferent fibers in the cat knee joint by antidromic nerve stimulation is inhibited by the μ-opioid, sufentanil [51].

Evidence that opioid receptors are also present on the surface of infiltrating immune cells is available in abundance [52-59]. These receptors have similar characteristics to the opioid receptors located on neurons [60]. Specific binding sites for opioids have been found on immune cells such as neutrophils and monocytes [61], [62], as well as on T-lymphocytes, platelets, and polymorphonuclear leucocytes (for review see [63]). The recent cloning of the three opioid receptor subtypes and the ability to raise antibodies to each receptor subtype now offer improved methods for locating receptor binding sites and will enable better identification of the cells which bear opioid receptors.

Possible mechanisms for anti-inflammatory effects of opioids

Against this background, the discovery that a κ-opioid agonist reduced inflammation in adjuvant arthritis raises interesting questions about the mechanisms and inflammatory mediators involved. The close spatial and functional association between nerves and immune cells [64, 65] and the presence of opioid receptors on both afferent fibers and immune cells suggest that neural and immune mechanisms are both likely to be involved.

Role of primary afferent neurons

Synovial joints are extensively innervated by afferent fibers which are immunoreactive for inflammatory neuropeptides (for review see [66]). These neuropeptides include SP, calcitonin gene related peptide (CGRP) and

somatostatin. SP is effective in generating the peripheral signs of inflammation, especially increased vascular permeability; in fact, SP receptors have recently been localized to the endothelial gap junctions in venules [67]. In some tissues SP also induces vasodilatation but in others (eg the dura mater) CGRP is the vasodilating peptide [68]. Although changes in SP concentrations are not always tightly linked to the degree of inflammation, clinical and experimental studies have suggested a contribution of afferent fibers and neuropeptides to the expression of arthritis [31, 66]. For example, there are "high-risk" joints in experimental arthritis and they contain the highest concentration of SP because they have a greater density of afferent fibers; further, infusion of SP into the rat knee for two days prior to disease induction, increased the severity of experimental arthritis [69]. The mechanism could involve the recently described SP receptors on Schwann cells and mast cells in close proximity to nerves [67]; their activation could, for example, increase direct exposure of the neuronal membrane to agents in the biophase through retraction of the Schwann cell processes. The fact that the development of adjuvant arthritis is less severe when sensory function is impaired by pre-treatment with the neurotoxin capsaicin [70, 71] is consistent with these ideas. However, this theory is still controversial: Ahmed and colleagues [72] reported that capsaicin was without effect in this respect. The argument for the nervous system involvement is reinforced by the findings of Donaldson *et al* [73], who found that as experimental arthritis develops, levels of mRNA for SP within the relevant dorsal root are increased. Furthermore, when rheumatoid arthritis develops in patients who have lower motor neurone damage as a result of poliomyelitis, the paralysed limbs are commonly wholly or partially spared the arthritis [74], clearly suggesting that the nervous system is a crucial factor in the development of this disease. The characteristic symmetrical nature of rheumatoid arthritis also argues that neuronal mechanisms are involved [75]. However, despite strong evidence linking the nervous system to pain and inflammation of arthritis, further work is required to determine the precise stage of the disease at which neuropeptides play a role, what CNS loci are involved, and how interfering with their actions might alter the course of arthritis.

In 1977, Jessell and Iverson suggested that opioids mediate a presynaptic inhibition of stimulus-evoked SP release from the central endings of primary afferent terminals via opioid receptors [76]. Consistent with this concept, others have found that opioids could inhibit SP release from the peripheral endings of primary afferent fibers [51, 77-79]. More specifically, Taddese *et al* [80] have found that opioids selectively reduce Ca^{2+} currents in the smallest nociceptive neurons (which would be responsible for what is known as "second pain") but not in larger nociceptive cells (which would be the basis of the sharp localized "first pain"). This diminished Ca^{2+} current would reduce transmitter release and could, therefore, be the basis of the presynaptic inhibition. In contrast, morphine application to slices of guinea pig brain was found to stimulate SP release [81]. The mechanisms, however, appear to be complex because high doses of the κ-agonist U50488H, were shown to inhibit SP release while low doses facilitated its release. In another study, low doses of morphine were inhibitory but high doses facilitated SP

release [82]. As mentioned above, and in support of these data, naloxone also has biphasic dose-dependent effects on adjuvant arthritis. It appears, therefore, that opioid modulation of SP release is concentration-dependent, and the resultant dose-response relationship is bell-shaped.

To further complicate matters, SP and opioid peptides are co-localized in peripheral terminals of primary afferent neurons [83]; when they are released, their interactions may be complex. For example, co-administration of very low doses of SP (10 to 100 pmol), intrathecally, with marginally effective doses of morphine sulfate, resulted in a markedly enhanced analgesic response (using the rat tail-flick test) [84]. Pre-treatment with naloxone abolished the hypoalgesia, indicating a mechanism mediated by opioid receptors. Thus, SP may enhance opioid analgesia by increasing release of opioid peptides within the CNS [84] and it is possible that a similar mechanism exists for the anti-inflammatory effects of opioids in the periphery.

Role of the immune system

Opioids are also known to exert effects on cells of the immune system. For example, morphine has variable immunosuppressive actions. It causes involution of such organs as the spleen and thymus, reduces the numbers of circulating lymphocytes, suppresses T-lymphocyte function, depresses the phagocyte and killing properties of monocytes and impairs antibody production [85]. It probably achieves these actions by inhibiting the release of cytokines like interleukin-1 (IL-1) and IL-2 from immune cells [62, 86, 87]. For a comprehensive treatment of this important topic, readers are referred to the excellent review by Bryant and Holaday [62]. To the extent that immuno-hyperactivity might be an important aspect of rheumatoid arthritis, the hypothesis that opioids might have a role in the treatment of established disease or in aborting developing arthritis is an attractive one. This action would presumably be through actions on opioid receptors which are located on the surface of immune cells.

What is the physiological role for opioid peptides in inflammation? There is now both, clinical [88] and experimental [49, 89-91] evidence that immune cells contain not only large amounts of endogenous opioid ligands, such as β-endorphin, dynorphin and the enkephalins, but also the relevant mRNAs [88]. These findings suggest that opioid peptides are synthesized within the immune cell and are then released following an inflammatory stimulus [86, 92]. The intrinsic mechanisms that trigger the release of immune cell-derived opioid peptides are unknown, but it has been found that cytokines [86] and peripheral corticotrophin-releasing factor (CRF) can cause their release [93]. Consistent with this hypothesis, naloxone produces exacerbation of local inflammation induced by subplantar injection of carrageenan or saline, presumably by antagonizing the local action of endogenous opioid peptides [94]. However, there are some discrepancies in the literature. For example, while opioids diminish T cell function, they may also enhance chemotaxis, adherence and mediator release from monocytes and macrophages [62]. Overall, it is clear that the endogenous opioid system has an important role in the physiological response to inflammation, reinforcing the potential significance of opioid drugs in RA.

The co-operative action between cytokines and opioid peptides is also worthy of consideration. This was clearly illustrated by an experiment in rats with unilateral inflammation induced by Freund's adjuvant. Intraplantar injection of either TNF-α or IL-6 induced a dose-dependent analgesia in the inflamed paw, which could be prevented by naloxone or opioid peptide antibody [92]. It was thus proposed that cytokines release opioid peptides (e.g. β-endorphin and/or enkephalins) from immune cells of the inflamed tissue, which then act on opioid receptors present on sensory nerve terminals resulting in antinociception [86, 92]. Thus, opioids can act as immunomodulators and may be important in the control of immune-inflammatory diseases like RA.

Neural-immune interaction

The experimental evidence already discussed highlights the likelihood of neuro-immune-interactions in the control of inflammation, ie the nervous and immune systems may have complementary functions which would be significant in arthritis. A schematic view of this complex system is presented in Figure 1, illustrating that SP activates the neutrophils and macrophages inducing the release of such cytokines as IL-1, IL-6 and TNF-α. In addition, SP stimulates degranulation of mast cells, producing a local release of histamine, serotonin and cytokines [95, 96]. Thus, there is bi-directional communication between neural and immune cells which is sustained by release of mediators from both sides. The net result is a positive feedback and maintenance of the inflammatory response.

A major point of neuro-immune convergence is in what is known as the hypothalamic-pituitary-adrenal (HPA) axis, a system of great importance in the Selye "Stress Reaction". Opiates have protean effects on immune function (notably depending upon whether administration is acute or chronic), significant aspects of which are focussed in the HPA axis [97]. Clearly there are μ-opioid receptors in the hypothalamic nuclei which, acutely, provoke release of CRF (corticotrophin-releasing factor) and thus ACTH and glucocorticoids in both stressed and unstressed animals, though the CRF-HPA system is inhibited when opioid exposure is chronic (for review see [98]). Each of these compounds has immune actions. CRH releases opioids from immune cells [91] and thereby induces analgesia through an action on peripheral afferent fibers [93]; it also has immunosuppressive actions [99]. ACTH and β-endorphin (which are released concomitantly from the anterior pituitary) have immuno-inhibitory actions [100, 101], while the end-products of this axis, the adrenal glucocorticoids, have been used clinically for their powerful immunosuppressive actions for years. The neural-derived cells of the adrenal medulla also produce opioids [102, 103] and postganglionic sympathetic neurones (of which the medullary cells are a special type) release a variety of inflammatory factors [104]. In turn, cytokines (in particular TNF-α, IL-1 and IL-6) stimulate secretion of CRF and arginine vasopressin (AVP); these two hypothalamic releasing factors synergize to evoke ACTH secretion. It is noteworthy that a subgroup of patients with RA have abnormalities in the HPA axis [105].

The role of the nervous system, particularly opioid transmitters, in the development and maintenance of inflammation (hence, of inflammatory disease), is thus of great potential significance. Therefore, peripherally selective opioids promise a real advance in the therapy of RA [10].

Peripherally Selective Opioids

It is now well established that opioids may produce antinociception by activating peripheral opioid receptors [25, 106, 107]. As already mentioned, intra-articular morphine produces pain relief following knee arthroscopy without systemic effects [42] or tolerance [108]. Such antinociception is more marked in inflamed tissue [109]. Walker's group have found that the κ-opioid agonist, U50488H, has analogous peripheral anti-inflammatory effects. When it is administered directly into an inflamed paw, at a dose that produces no systemic effects, it has a potent anti-inflammatory action; to achieve the same result with systemic administration requires at least four times the dosage, with the concomitant risk of systemic effects [10]. These peripheral analgesic and anti-inflammatory effects could have several explanations. They may be due to increased synthesis of opioid receptors and their axonal transport to the peripheral terminals [91] [110]. Alternatively, they could be due to an inflammation-induced disruption of the perineural barrier and subsequent facilitated access for the opioid agonist to pre-existent (but inaccessible or "sleeping") opioid receptors on the peripheral nerves [111]; the SP receptors on Schwann cells (referred to above) may have a similar effect [67].

Opioids administered directly into the inflamed site have either pro- or anti-inflammatory effects in the formalin-induced extravasation model of inflammation [112]. In this model, μ- and δ-agonists attenuated plasma extravasation in both the early and late phases, whilst κ-agonists enhanced extravasation but only in the early phase. All of these effects were blocked by a peripherally selective opioid antagonist [112]. Few peripheral effects on δ-receptors have been reported previously, but these workers also found that the δ-agonist D-Pen-D-Pen-enkephalin (DPDPE) inhibited plasma extravasation in formalin-induced inflammation.

In rheumatoid arthritis, a potentially successful strategy to ameliorate the adverse side effect profile of opioids is to confine their access to the peripheral nervous system. Selective opioid agonists that can be absorbed through the gut, ie are orally active, but do not cross the blood brain-barrier would simultaneously eliminate the need for multiple injections and avoid CNS toxicity. This selectivity can, in principle, be achieved by changing the chemical structure of these drugs but early attempts to reduce their access to the brain by increasing hydrophilicity, as in quaternary ammonium analogs, also decreased their potency (for review see [25]). However, a novel kappa agonist, EMD-61753 (asimadoline, Merck KGaA), that includes a hydrophilic and hydrophobic portion in the molecule, has now been developed. It has very limited ability to cross the blood-brain barrier and inhibits the plasma extravasation which is evoked by electrical stimulation of the saphenous nerve, an anti-inflammatory action which is reversible by administration of the kappa antagonist, nor-BNI, directly into the inflamed site (at doses insufficient to inhibit systemic opioid effects) [113].

Figure 1. Schematic illustration of neuro-immune-interactions in the control of inflammation.
Rheumatoid arthritis is triggered by the formation of an immune complex between a T cell and an antigen-presenting cell. This complex stimulates the release of substance P (SP) from peripheral sensory neurons as well as the release of interferon-g (IFN-γ) from the T cell. Substance P can then stimulate macrophages to release cytokines (tumor necrosis factor-α (TNF-α) and interleukins (IL's)), neutrophils to release proteases and mast cells to release TNF-α, serotonin (5HT) and histamine. The serotonin and histamine can also stimulate release of SP from nerve endings. The release of IFN-γ from the T cell not only stimulates the release of the cytokines TNF-α and IL's from macrophages but, together with these cytokines, feeds back on the T cell to stimulate the release of more IFN-γ. TNF-α and IL's then act on synoviocytes to release proteases. Together with the proteases released from the neutrophil, these enzymes cause irreversible cartilage and bone erosion.
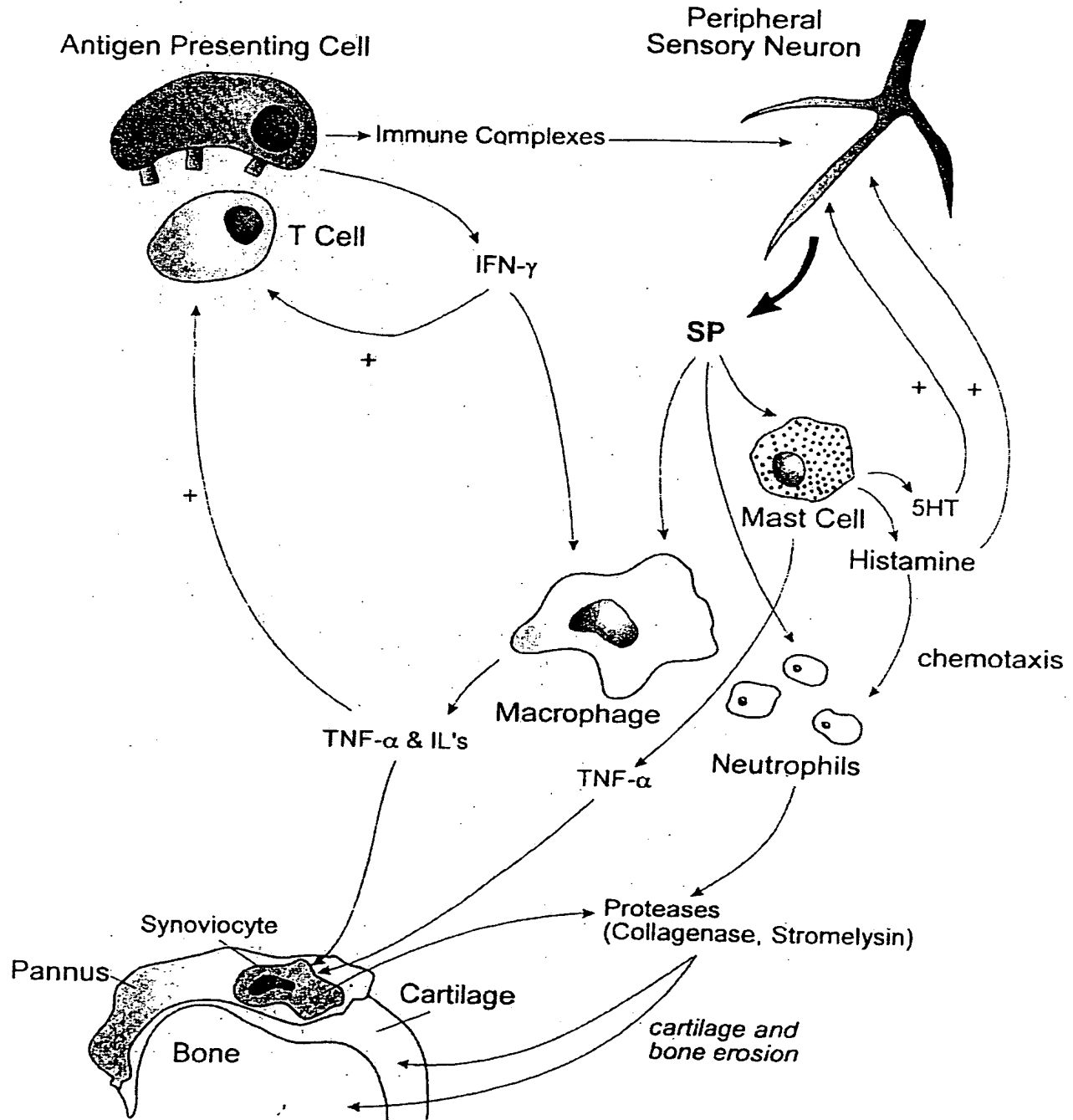
Even more relevant, experiments by Walker's group have shown that EMD-61753 is a very potent anti-arthritic compound (with a mean effective dose – 1 mg/kg s.c.) and is able to reduce the severity of adjuvant arthritis in rats by as much as 50% after both parenteral and oral administration [114]. Such novel opioid drugs, which are essentially without central side effects, herald radical new treatments for RA sufferers.

Summary

Opioid drugs are not currently used in the treatment of RA, partly because of their range of side-effects and because their anti-inflammatory (as opposed to analgesic) actions have been largely unrecognized. These drugs, in fact, have important anti-inflammatory effects at peripheral sites (e.g. on peripheral terminals of primary afferent nerves and on various immune cells), presumably involving effects on the release or actions of pro-inflammatory neuropeptides and cytokines from immune cells. The use of peripherally selective opioids in experimental arthritis produces clear anti-inflammatory results without central complications. This new approach has considerable clinical promise in the widespread and debilitating disease of RA.

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